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# A functional dissection of the yeast CCR4 protein and identification of associated factors

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**A functional dissection of the yeast CCR4 protein and  
identification of associated factors**

**Draper, Michael Preston, Ph.D.**

**University of New Hampshire, 1994**

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**A FUNCTIONAL DISSECTION OF THE YEAST CCR4 PROTEIN AND  
IDENTIFICATION OF ASSOCIATED FACTORS**

by

**Michael Preston Draper  
B.A., Saint Anselm College, 1988  
M.S., University of Connecticut, 1990**

**DISSERTATION**

**Submitted to the University of New Hampshire  
in Partial Fulfillment of  
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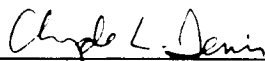
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in


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This dissertation has been examined and approved.



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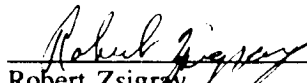
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## DEDICATION

Dedicated to my parents

Robert and Louise Draper



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## TABLE OF CONTENTS

DEDICATION . . . . .	iii
ACKNOWLEDGMENTS . . . . .	iv
LIST OF TABLES . . . . .	vi
LIST OF FIGURES . . . . .	vii
ABSTRACT . . . . .	viii
GENERAL INTRODUCTION . . . . .	1
I. FUNCTIONAL DISSECTION OF THE YEAST CCR4 PROTEIN . . .	19
Introduction . . . . .	19
Materials and Methods . . . . .	20
Results . . . . .	21
Discussion . . . . .	25
II. IDENTIFICATION OF FACTORS ASSOCIATED WITH CCR4 . . .	26
Introduction . . . . .	26
Materials and Methods . . . . .	28
Results . . . . .	33
Discussion . . . . .	55
GENERAL DISCUSSION . . . . .	58
REFERENCES . . . . .	62
APPENDIX . . . . .	69

## LIST OF TABLES

Table 1. Yeast Strains Used in Chapter II . . . . .	29
Table 2. Phenotypes of a <u>yCAF1</u> disruption . . . . .	50
Table 3. Mutations in <u>yCAF1</u> affect the activation potential of various activators . . . . .	52

## LIST OF FIGURES

Figure 1. Regulatory factors identified in controlling the yeast <u>ADH2</u> gene . . .	7
Figure 2. The role of ADH II in carbon metabolism . . . . .	8
Figure 3. The leucine rich repeat is required for LexA-CCR4 transcriptional activation . . . . .	22
Figure 4. Transcriptional Interference assays with LexA-CCR4 derivatives . . . .	24
Figure 5. Interaction of mCAF1 with CCR4 . . . . .	35
Figure 6. mCAF1 co-immunoprecipitates with CCR4 . . . . .	37
Figure 7. Alignment of the CAF1 proteins from yeast, mouse, <i>C.elegans</i> and <i>Arabidopsis</i> . . . . .	40
Figure 8. Identification of the domain in CCR4 responsible for the interaction with CAF1 . . . . .	42
Figure 9. yCAF1 specifically interacts with CCR4 as judged by immunoprecipitation . . . . .	45
Figure 10. yCAF1 is complexed with the 185 and 195 kDa proteins . . . . .	46
Figure 11. Wild type yCAF1 is associated with CCR4 . . . . .	48
Figure 12. LexA-yCAF1 can function as an activator . . . . .	54

## ABSTRACT

### A FUNCTIONAL DISSECTION OF THE YEAST CCR4 PROTEIN AND IDENTIFICATION OF ASSOCIATED FACTORS

by

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University of New Hampshire, May, 1994

The yeast CCR4 protein is required for the expression of a number of genes involved in non-fermentative growth, including glucose repressible ADH2, and is the only known suppressor of mutations in the SPT6 and SPT10 genes, two genes which are believed to be involved in chromatin maintenance. It is shown here that CCR4 is able to activate transcription when fused to a heterologous DNA binding domain. The transcriptional activation ability of CCR4, in contrast to many other activators, was glucose regulated. Two activation domains were identified, one of which was glucose responsive and encompassed a glutamine-proline rich region similar to that found in other eukaryotic transcriptional factors. The two transactivation regions, when separated from the leucine-rich repeat and the C-terminus of CCR4, were unable to complement a defective ccr4 allele, suggesting that the leucine-rich repeat and the C-terminus make contacts that link the activation regions to the proper gene context.

Also reported here is the identification of a protein from mouse (mCAF1) which

was capable of interacting with and binding to the yeast CCR4 transcriptional regulatory complex. The mCAF1 protein was shown to share significant similarity with proteins from human, *C.elegans*, *Arabidopsis*, and yeast. Both the yeast and *C. elegans* homologs of mCAF1 were shown to interact with CCR4 *in-vivo*. Disruption of the yCAF1 gene in yeast gave phenotypes and defects in transcription similar to those seen with disruptions of CCR4. yCAF1 when fused to the LexA DNA binding domain also functioned as a strong activator of transcription in yeast.

Immunoprecipitation of yCAF1 revealed that it was complexed with the 185 and 195 kDa species previously shown to associate with CCR4. The binding of CCR4 and yCAF1 to the 185 and 195 Kda proteins was not interdependent. These data indicate that the transcriptional regulatory complex composed of CCR4, the 185 and 195 kDa proteins and yCAF1 make up part of an evolutionarily conserved complex involved in transcriptional control.

## GENERAL INTRODUCTION

Transcription initiation by eukaryotic RNA polymerase II requires a variety of protein factors in order to occur in a spatially and temporally regulated manner. These factors can be divided into different groups. First the general initiation factors, along with RNA polymerase II, are required at most promoters. The well characterized general factors implicated in transcription initiation on TATA containing promoters include TFIIA, TFIIB, TFIID, TFIIIE, TFIIF, TFIIH and RNA polymerase II (reviewed in Weinmann, 1992). The polypeptide compositions of these factors are well established and it is clear that the structure and function of these factors, like RNA polymerase II, are conserved throughout the eukaryotic world. Even RNA polymerases I, II, and III are similar in subunit structure and function in higher and lower eukaryotes (Young, 1991). The yeast RNA polymerase II factors b, d, and e are homologous in structure and function to mammalian factors TFIIH, TFIID and TFIIB respectively (Peterson et al., 1990; Thompson et al., 1993). This conservation of structure would imply that the mechanism by which gene specific activators function to effect the formation of a stable complex containing these factors has been conserved. This has been borne out by the observation that many transactivator proteins from higher eukaryotes function in yeast (Ptashne, 1988; Berger et al., 1992; Kelleher et al., 1992).

Many regulatory proteins confer activation or repression in a more gene specific

manner. These proteins are capable of binding to short DNA stretches located in the 5' non-coding regions, usually several hundred nucleotides upstream of the TATA box. These DNA regulatory elements are referred to as enhancers or UAS elements (upstream activation elements) in the case of an activator binding to them and URS elements (upstream repressor element) in the case of a repressor binding. *In vitro* studies have revealed that the general components possess the ability to interact with factors bound to the regulatory elements. For example, the TFIIB activity in a HeLa cell nuclear extract is specifically retained on a column containing an acidic activation region and recombinant TFIIB behaves in a similar manner (Lin et al., 1991). In a two-hybrid type screen an acidic activation domain from *E.coli* has been shown to interact with a LexA-TFIIB fusion (Guarante, personal comm.). The *in vitro* data would argue for a direct interaction between an activation domain and TFIIB. However, an important property of eukaryotic transcriptional activators is that they can cooperate with one another to stimulate transcription synergistically (Ptashne, 1988). These data would suggest that during preinitiation complex assembly more than one transcription component may be contacted by the bound activators. This model is consistent with experiments demonstrating that factors such as TBP and TAF110, in addition to TFIIB are capable of interacting with activating regions (Stringer et al., 1990; Hoey et al., 1993; Dynlacht et al., 1991; Zhou et al., 1992; Tanese et al., 1991). However, not all activators make direct contacts to the core transcriptional machinery.



These other factors which are mediating the signal from the DNA bound transactivator proteins have been called co-activators or adaptors and represent the third class of factors. Mutations in genes have been isolated which appear to mediate the activation potential of activators. One such pair of genes are the ADA2 and ADA3 genes in yeast (Berger et al., 1992; Pina et al., 1993)(reference to a gene in yeast is denoted by underlining the name whereas protein names are not underlined; uppercase letters refers to the wild type allele or protein whereas lowercase to the mutant allele or protein). These adaptor proteins are quite possibly associated with the general factors and therefore may actually fall into the category of a general factor themselves. For instance some of the TATA binding protein-associated-factors (TAFs) appear to function as co-activators by providing a functional link between sequence specific regulators and TATA binding protein (TBP) (Zhou et al., 1992; Hoey et al., 1993; Tanese et al., 1991). In yeast there are specific proteins designated as TAFs (J. Jaehning personal comm.). However, there also exists another group of proteins known as the SRBs which may or may not be classified as TAFs. These SRB proteins have been identified as being associated with yeast RNA polymerase II, specifically with the RNA polymerase II carboxy-terminal domain (CTD) (Thompson et al., 1993). The CTD is a highly conserved feature of the largest subunit of RNA polymerase II. Deletion mutations that remove most or all of the CTD are lethal to cells (Bartolomei et al., 1988) and in yeast there is a progressive loss in the ability to induce transcription of specific genes such as GAL10 as the CTD is truncated (Scafe et al., 1990). It has been suggested that this large

multi-subunit complex containing TBP could act as a central processor to receive and act on both positive and negative transcriptional regulatory signals (Allison and Ingles, 1989; Thompson et al., 1993).

These data suggest that the role of regulatory factors must be to stimulate basic factor interactions, by acting directly on the pre-initiation complex or through co-activators, that are otherwise limiting. Such limitations could be imposed by non-consensus binding sites, by competition between genes for a limited pool of general factors or by negative constraints such as chromatin structure.

Finally, histones and other protein factors which are involved in chromatin structure play a role in regulating transcription (Pugh and Tjian, 1991). The importance of chromatin structure in gene regulation has been demonstrated in the reorganization of structure found upon activation of the PHO5 gene (Felsenfeld, 1992). Similarly, the yeast  $\alpha 2$  repressor functions by ordering a short array of nucleosomes to block access of TFIID to the TATA box (Shimizu et al., 1991). Workman and colleagues have shown that reconstitution of chromatin on a template *in vitro* strongly suppresses the subsequent activity of the template for transcription with basal factors (Workman et al., 1991). In yeast, nucleosome depletion caused by inhibition of histone synthesis results in high levels of transcription by many genes even in the absence of activators that are normally required for gene expression (Grunstein, 1990).

Transcriptional activation experiments suggest that there are two kinds of mechanisms for disruption of chromatin structure. The first mechanism is a dynamic

process in which competition between trans-activator proteins and histones does not require DNA replication. In this case binding sites for critical protein factors would be made accessible or other trans-acting factors would be able to destabilize or displace a histone octamer making a site available. The second mechanism is the pre-emptive model in which chromatin changes would occur during replication. These changes would establish a nucleosome free region allowing trans-activator proteins to act (Felsenfeld, 1992).

Recently, genetic and molecular evidence has been presented suggesting that the trans-activator proteins SNF2 and SNF5 function by antagonizing repression mediated by nucleosomes. First, the transcriptional defects in strains lacking these SNF genes are suppressed by a deletion of one of the two sets of histones H2A and H2B. Second, chromatin structure is altered in snf2 and snf5 mutants, and this defect is suppressed by the deletion of H2A and H2B (Hirschhorn et al., 1992). Furthermore activation by a LexA-GAL4 or a LexA-Bicoid fusion has been shown to be dependent upon the SNF2 and SNF5 proteins (Laurent and Carlson, 1992). It is possible that the SNF2 and SNF5 proteins function coordinately with activators to alleviate nucleosomal repression.

Genetic studies in the yeast *Saccharomyces cerevisiae* have contributed to our understanding of the complexities of transcription initiation by RNA polymerase II. In many cases, these studies have progressed from the isolation of mutations affecting the transcription of certain genes to the isolation of extragenic suppressors of the initial mutations. These elaborate genetic analyses have identified genes essential or

important for transcription *in vivo*.

Genetic analysis of the alcohol dehydrogenase II system in *Saccharomyces cerevisiae* has identified factors that are unique to the transcriptional regulation of the alcohol dehydrogenase two gene (ADH2), as well as factors that are common to the regulation of a number of other genes in yeast (Figure 1). The yeast ADH2 gene acts to oxidize ethanol to acetaldehyde in a NAD<sup>+</sup> linked reaction and is therefore the initial step in ethanol utilization (Figure 2) (Ciriacy, 1975). Expression of ADH2 is regulated on the basis of carbon source. When yeast are switched from a fermentable to a non-fermentable carbon source, ADH2 transcription is increased 500-fold in cells grown in medium with little or no glucose (Denis et al., 1981).

The structural gene for ADH2 has been cloned and sequenced (Williamson et al., 1981; Russell et al., 1983). Two upstream activation sequences (UAS elements) are located upstream of the TATA element. The first element UAS1 is a 22 base pair dyad located from -291 to -271 relative to the start of translation. The second element, UAS2, is located 6-base pairs upstream from UAS1 ranging from approximately -297 to -335. All of these elements are necessary for full ADR1 activation of the ADH2 gene (Yu et al., 1989). Downstream of the UAS elements lies a poly (dA)<sub>20</sub> tract (Figure 2).

Several transacting factors affecting the transcriptional level of the ADH2 gene have been identified (Ciriacy, 1975, 1977; Denis, 1984). The first of these factors is the positive effector ADR1. The gene encoding the ADR1 protein was cloned (Denis and Young, 1983) and when sequenced found to encode a protein of 1323 amino acids

Figure 1. Regulatory factors identified in controlling the yeast ADH2 gene.

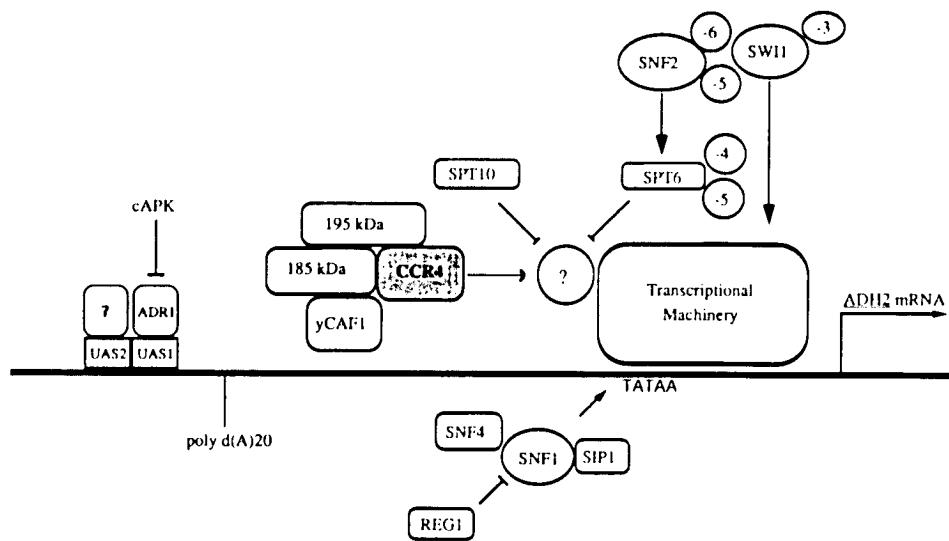
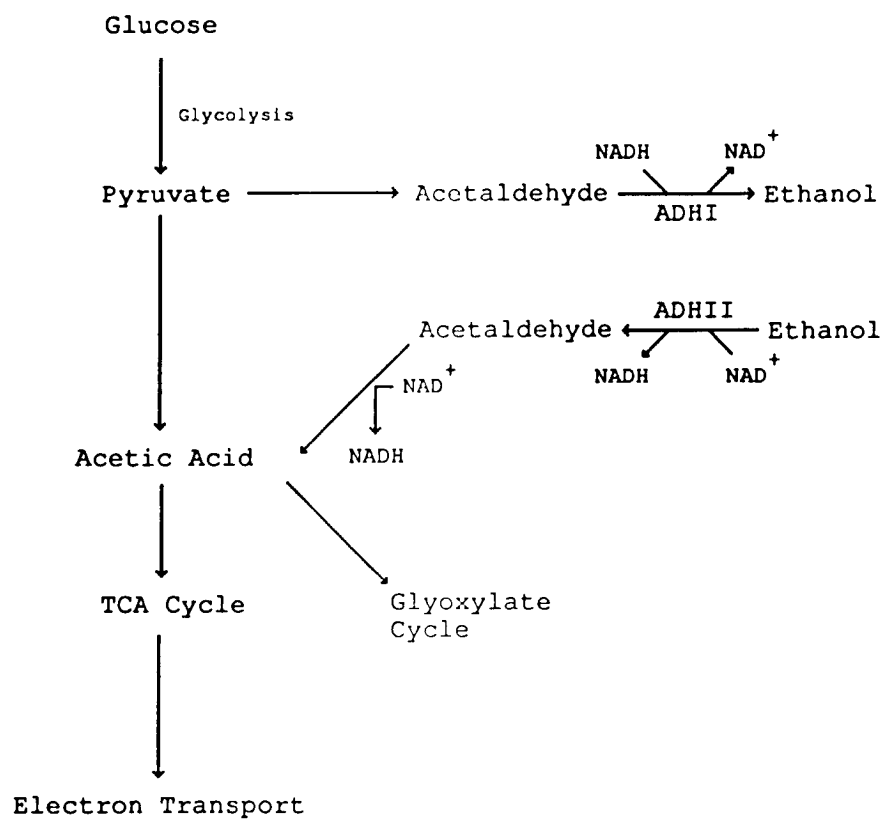


Figure 2. The role of ADH II in carbon metabolism.



with a molecular mass of 151 kDa (Hartshorne et al., 1986). The protein contains several identifiable functional domains (Cook et al., 1994). One of these regions (amino acids 99-155) contains two DNA-binding zinc fingers (Hartshorne et al., 1986). ADR1 uses these zinc fingers to bind to the UAS1 element located in the ADH2 promoter (Yu et al., 1989).

The negative regulatory elements SPT10 and SPT6 along with the positive effector CCR4 appear genetically to constitute a regulatory pathway separate from that of the ADR1 pathway. SPT10 and SPT6 were identified as mutations that allow escape of the ADH2 gene from glucose repression. Strains containing mutations in these genes were identified by screening cells for increased ADH II expression and thus the ability to grow in the presence of glucose and the respiratory inhibitor antimycin A (Denis, 1984). Mutations in CCR4 were subsequently identified by their ability to suppress the increased ADH2 expression on glucose caused by the spt6 or spt10 allele. Northern analysis revealed that the SPT6, SPT10 and CCR4 gene products act at the transcriptional level to control ADH2 expression. (Denis, 1984; Denis and Malvar, 1990).

The ccr4, spt6 and spt10 alleles are pleiotropic in their effects. Strains harboring a mutation in CCR4 show a temperature sensitive phenotype on non-fermentative medium at 37°C (Denis and Malvar, 1990) and a cold sensitivity at 16°C on fermentative media (Draper et al., 1994). Mutations in CCR4, have also been shown to affect the derepression of other non-fermentative genes such as malate dehydrogenase, malate synthase, and isocitrate lyase (Denis, 1984). Mutations in

either SPT10 or SPT6 affect cell morphology. In cells containing an spt10 allele a mutation in CCR4 has been shown to suppress the altered cell morphology. SPT6 mutations have the added phenotype of conferring temperature sensitive growth. This phenotype is not suppressible by a ccr4 allele (Denis and Malvar, 1990).

The SPT6 and SPT10 genes have been identified as being important components of the transcriptional regulatory machinery in a number of other systems. The SPT (suppressors of TY) genes were originally identified as mutations that overcome the effects of delta insertions at the his4-912 delta locus (Clark-Adams and Winston, 1987; Winston et al., 1984). Delta sequences are the approximately 300 bp terminal repeats of the yeast retrotransposon Ty. They are often left behind following transposition of a Ty element. Insertion mutations in the 5' regions of genes caused by Ty elements or delta sequences often abolish or alter transcription of the adjacent gene. These effects are believed to be due to interference or competition by transcription signals in the Ty or delta with those of the adjacent gene. In the strains containing the spt6 and spt10 alleles transcription initiating in Ty elements or delta sequences is reduced and transcription of the adjacent gene is restored. Therefore SPT6 and SPT10 function somehow to affect competition between transcription signals in the Ty and delta elements and those of adjacent genes. In addition SPT6 and SPT10 have been identified as factors involved in the regulation of a number of other genes including, the HO endonuclease and SUC2 (Swanson et al., 1990). Mutations in CCR4 suppress the effects that spt10 and spt6 have in overcoming insertions at the his4-912 delta or adh2-601 delta promoters (Denis and Malvar,



1990).

The CCR4, SPT6 and SPT10 gene products act at or near the TATA element of the ADH2 promoter (Denis and Malvar, 1990). Strains with deletions or disruptions upstream of the ADH2 promoter continue to show elevated ADH II activity under repressed conditions in a spt10 or spt6 background (Denis and Malvar, 1990). Furthermore, mutations in CCR4 are still able to suppress this increased ADH2 expression (Denis and Malvar, 1990).

The genes for SPT6 (Swanson et al., 1990) and SPT10 (Natsoulis et al., 1994) have been cloned and sequenced. SPT6 encodes a nuclear protein that has an estimated mass of 170 kDa and contains a highly acidic amino terminus (Swanson et al., 1990). Increasing and decreasing the dosage of SPT6 results in suppression of the his4-912 delta phenotype. This suppression is similar to what is seen when histone dosage is varied (Clark-Adams et al., 1988). Further, proteins with highly acidic regions, such as those found in the SPT6 protein, have been implicated in chromatin binding (Earnshaw, 1987). It may be that SPT6 plays a role in chromatin structure.

Further evidence supporting the SPT6 chromatin connection comes from genetic studies identifying SPT6 as a suppressor of mutations in SNF2/SWI2 (hereafter referred to as SNF2) and SNF5. SNF2 and SNF5 are positive effectors of transcription and have been implicated as participating in a common function possibly as a heteromeric complex (Laurent et al., 1991). In addition, although the SNF2 and SNF5 proteins have not been shown to bind DNA they appear to play a direct role in

transactivation because LexA-SNF2 and LexA-SNF5 protein fusions can activate transcription of a reporter gene containing LexA operator sites (Laurent et al., 1990; Laurent and Carlson, 1992). Other results indicate that the SNF2 and SNF5 proteins activate transcription by altering chromatin structure (Hirschhorn et al., 1992). This mechanism of regulating gene expression by remodeling the local chromatin structure of a promoter does not seem to be unique to yeast, since SNF2 has been shown to have extensive similarity to proteins in other eukaryotes including the *Drosophila* transactivator protein *brahma* (Tamkun et al., 1992). The *brahma* gene was identified as being necessary for activation of some homeotic genes (Tamkun et al., 1992). The striking homology and the fact that both genes are involved in transcriptional activation would suggest a conservation of the mechanism by which both are acting. Perhaps even more interesting than this shared structural similarity is the observation that both have extended homology to ATP-dependent DNA helicases (Laurent et al., 1992) and SNF2 has recently been shown to possess DNA-stimulated ATPase activity (Laurent et al., 1993). This finding suggests the possibility that the SNF-SWI complex functions to open up the chromatin structure and so regulate the accessibility of binding sites for other proteins.

LexA-SNF2 or LexA-SNF5 fusions can activate transcription of a reporter gene containing upstream LexA operator sites (Laurent et al., 1991). This observation not only implicates the SNF-SWI complex in having a direct role in activation but also suggests that there must be a mechanism to target this complex to the proper coordinates. In this respect the complex would act as a co-activator communicating

between the primary activation protein and the initiation complex.

Mutations in the yeast SPT6 gene have been shown to suppress defects in the SNF2, SNF5, and SNF6 genes (Neigborn et al., 1986). Defects in SPT6 result in phenotypes similar to those observed for alteration in histone abundance. Such similarities suggest a role for SPT6 in maintaining chromatin structure. Further, there is an interdependence of the SNF2, SNF5 and SNF6 protein in transcriptional activation. Activation by a DNA bound LexA-SNF5 fusion protein was greatly reduced in a snf2 and snf6 mutant background. An spt6 mutation which suppresses transcriptional defects caused by snf2, restored activation by LexA-SNF5 in a snf2 mutant background (Laurent et al., 1991). Mutations in spt6 also restored the ability of the LexA-SNF2 protein to activate in a snf5 background (Laurent et al., 1991). Moreover, it is worth noting that the SNF2,5,6 proteins not only function globally in yeast but also assist the heterologous activator Bicoid from *Drosophila*. Both a LexA-GAL4 and a LexA-Bicoid fusion were dependent upon SNF2 and SNF5 for normal activation of a target gene containing LexA operator sites (Laurent and Carlson, 1992). Once again this supports the notion that the basic machinery and the mechanisms by which this machinery functions have been conserved evolutionarily.

The release of genes from repressed states due to mutations in the SPT6 and SPT10 genes would suggest that they are negative regulators of transcription. These data coupled with the above observations would suggest that SPT6 and SPT10 may be functioning to keep chromatin in a repressed state and work in an antagonistic manner with the SNF and SWI proteins.

The CCR4 gene has been cloned and sequenced (Malvar et al., 1992). The gene encodes a protein with an estimated mass of 94.6 kDa. This is in agreement with the identified size of CCR4 as 96 kDa by SDS PAGE. The predicted amino acid sequence revealed several areas of interest. First there are five leucine-rich tandem repeats in the middle of the protein (residues 350-467). The motif is characterized by the consensus sequence P-X X - O X X L X X L X X L X X N X O X X O where X=any residue, O=aliphatic L, I or V. This region shares similarity with a family of proteins displaying similar leucine-rich motifs (Malvar et al., 1992). Leucine rich-repeat motifs have been shown to be involved in heterologous protein-protein interactions. Deletions of single or multiple copies of the repeats found in human placental ribonuclease inhibitor disrupts the inhibitors ability to bind and inactivate ribonuclease (Lee and Valee, 1990). The crystal structure of porcine ribonuclease inhibitor has recently been refined at 2.5 Angstrom resolution (Kobe and Deisenhofer, 1993). Individual repeats constitute  $\beta$ -sheet- $\alpha$ -helix structural units that probably also occur in other leucine-rich repeat containing proteins. This structure appears much like a horseshoe with the binding site for ribonuclease thought to be on the inner surface of the horseshoe. It was proposed that the non-globular shape of the structure and the exposed face of the parallel  $\beta$ -sheet may explain why leucine-rich repeats can achieve strong protein-protein interactions. Deletion of two or three repeats within CCR4 yielded a protein incapable of complementing a ccr4 mutation (Malvar and Denis, 1990). At least three proteins are capable of interacting with CCR4 and the leucine rich repeat is necessary and sufficient for interaction with two



from the fact that a LexA-CCR4(1-345) will strongly activate transcription of a down-stream reporter. However, it should be noted that the glutamine stretch in CCR4 is not identical to that found in Sp1. Moreover, at the present time this portion of CCR4 appears to be partly dispensable as a deletion of residues 14-209 is still able to complement a strain carrying a non-functional ccr4 allele.

The C-terminal region of CCR4 contains a region displaying homology to the xylose isomerase enzyme, specifically a domain shown to be involved in the binding of a metal ion (Jenkins et al., 1992). The region of CCR4 encompassing this domain is essential to CCR4 function. Mutations which truncate CCR4 at amino acid 668 are non-functional (D. Audino, personal communication). This portion of the protein may play a direct role in protein-protein interaction or may simply be involved in maintaining proper structure.

The orchestration of regulating gene expression is done by numerous factors as exemplified by the ADH2 system. Genetically it is possible to discern numerous pathways involved in the regulation of ADH2. One of these pathways involves the SNF/SWI general factors and may also involve the SPT6 and SPT10 proteins as well as CCR4. Evidence to date indicates however, that SPT10 and SPT6 in exerting control of transcription at ADH2 act through a factor that regulates CCR4 function but does not regulate CCR4 expression, control its activity, or physically interact with it. Rather SPT6 and SPT10 appear to act through a factor that requires CCR4 for function. Further examination of the relationship between the SNF/SWI proteins and CCR4 has shown that CCR4 protein expression is not affected by the SNF/SWI

components and nor does CCR4 co-immunoprecipitate with them. These data coupled with the different effects of mutations in CCR4 as compared to those for SNF2 and SNF5 on an ADH2 promoter mutation suggest that CCR4 acts by a separate mechanism (Denis et al., 1994).

While genetic analysis has revealed to us a wealth of information regarding some of the factors involved in ADH2 regulation it is clear that much is still not understood concerning the mechanisms and relationships between these factors. It was with a wish to better understand the function of one of these factors, CCR4, that this work was undertaken. The focus of this dissertation centered around two research goals. The first was to better define the function of CCR4 in transcriptional activation. To do this, LexA-fusions of CCR4 were constructed. This work allowed us to examine the function of CCR4 in a more defined system. Deletion analysis allowed mapping of functional domains based on the ability to activate and complement a ccr4-10 mutant allele. These LexA fusions also proved useful in examining the ability of CCR4 to bind DNA, and in the analysis of proteins interacting with the leucine-rich repeat of CCR4. This study is presented in Chapter 1 and in a manuscript by Draper et al. which can be found in Appendix A.

The second research goal was to identify the genes for proteins encoding CCR4 associated factors. To do this work a genetic screen, the interaction trap, was utilized. Results from this analysis revealed that CCR4 was able to complex with a protein from mouse which shares a high degree of structural similarity with proteins from a number of other organisms including yeast. The yeast homolog of this mouse

protein was shown to be a CCR4 associated factor. Further, a number of other genes in yeast were identified as being potential CCR4 associated factors. These are currently being analyzed further and are therefore not presented here. The identification and further characterization of the CCR4 associated factors will hopefully be useful in gaining more insight into the mechanistic function of this complex of proteins. This study is presented in Chapter 2.



## CHAPTER ONE

### I. FUNCTIONAL DISSECTION OF THE YEAST CCR4 PROTEIN

#### Introduction

In order to more fully understand the role of CCR4 in transcription and to begin to localize the functional domains of CCR4, various LexA-fusions of CCR4 were constructed. The construction of chimeric proteins containing the DNA binding domain of the bacterial LexA repressor protein (Brent and Ptashne, 1985) or the DNA binding domain of the yeast GAL4 activator protein (Keegan et al., 1986) has been one way of analyzing the transcriptional activation function of proteins in a defined context. LexA fusion proteins typically contain either the LexA amino-terminal DNA binding domain (LexA-1-87) or the complete protein (LexA-1-202) which also includes a dimerization domain. Like many DNA binding proteins native LexA binds as a dimer to an operator that consists of two dyad symmetric half-sites (Brent and Ptashne, 1986). LexA derivatives are assayed for transcriptional activation by using reporter genes that carry one or more LexA operators upstream of the transcription start site of a gene such as lacZ. For a non-activating derivative, DNA binding can be assayed by using a repression blocking assay, in which binding of the LexA derivative to an operator sequence located between an upstream activation site and the

transcription start site of a reporter gene diminishes its transcription (Keegan et al., 1986). This assay examines whether the absence of activation is a result of poor binding to DNA.

The ability of LexA-CCR4 fusions to complement the phenotypes of a ccr4 mutation can yield information that can be compared to data gathered using other deletions and truncations of CCR4. Further, the ability of a particular LexA-CCR4 derivative to activate can be correlated with its ability to complement. In addition, the LexA moiety acts as a convenient epitope tag which can be used to detect levels of the various fusion proteins by Western analysis or by immunoprecipitation.

The establishment of a genetic relationship between the SNF/SWI, SPT6, SPT10 and CCR4 genes led us to examine the ability of CCR4 to activate when fused to LexA. The majority of these data can be found in Appendix A of this thesis. A small amount of data which we felt was not applicable to the manuscript can be found in the short results section which follows. The results in Appendix A dealing with proteins binding to CCR4 was done in the main part by Hai-Yan Liu and DNA binding assays were performed by Stephen Mosley.

## Materials and Methods

Yeast strains See Appendix A.

Growth conditions and assays See Appendix A.

### Plasmid Constructions

The deletions within the leucine-rich repeat were constructed by removing an ApaI-BamHI fragment from the full-length LexA-CCR4 fusions (See Appendix A) and replacing this with an ApaI-BamHI fragment from the YRp7-3.5a derivatives containing the deletions of the leucine-rich repeats (Malvar et al., 1992). The ApaI fragment containing the coding sequence for amino acids 13-209 was then replaced.

## Results

### **The leucine-rich repeats of CCR4 are required for activation function**

A LexA-CCR4(1-837) fusion was able to activate transcription in a glucose repressed manner (See Appendix A). Previous deletion analysis indicated that the leucine-rich repeats of CCR4 were required for CCR4 activation at the ADH2 locus (Malvar and Denis, 1992). We investigated the role of the leucine-rich repeat domain in LexA-CCR4 activation potential by analyzing LexA-CCR4 fusions in which nearly three repeats, amino acids 393-456, two complete repeats residues 393-437, and sequences N-terminal to the repeats and including two repeats (residues 217-394) were removed. We found that the leucine-rich repeats were essential for CCR4 to function as a transcriptional activator irrespective of the carbon source (Figure 3). Western analysis indicated that these deletions did not affect the abundance of the LexA-CCR4 fusions (data not shown). The leucine-rich repeat region alone (LexA-CCR4-330

Figure 3. The leucine rich repeat is required for LexA-CCR4 transcriptional activation.

		Beta-Gal Activity		YEP-Etoh Growth 37°
		Glucose	Ethanol	
LexA 1-87		<1.5	<1.5	—
LexA 1-202		<1.5	2.9	—
LexA 1-87	1 ————— 837	9.5	89	+
LexA 1-202	—————	2.9	39	+
LexA 1-87	1 ————— 392 438 ————— 837	<1.5	<1.5	—
LexA 1-202	————— 392 438 —————	<1.5	<1.5	—
LexA 1-87	1 ————— 392 457 ————— 837	2.2	<1.5	—
LexA 1-202	————— 392 457 —————	1.5	1.5	—
LexA 1-87	1 ————— 218 ————— 395 ————— 837	1.6	1.5	—
LexA 1-202	————— 218 ————— 395 —————	<1.5	1.8	—

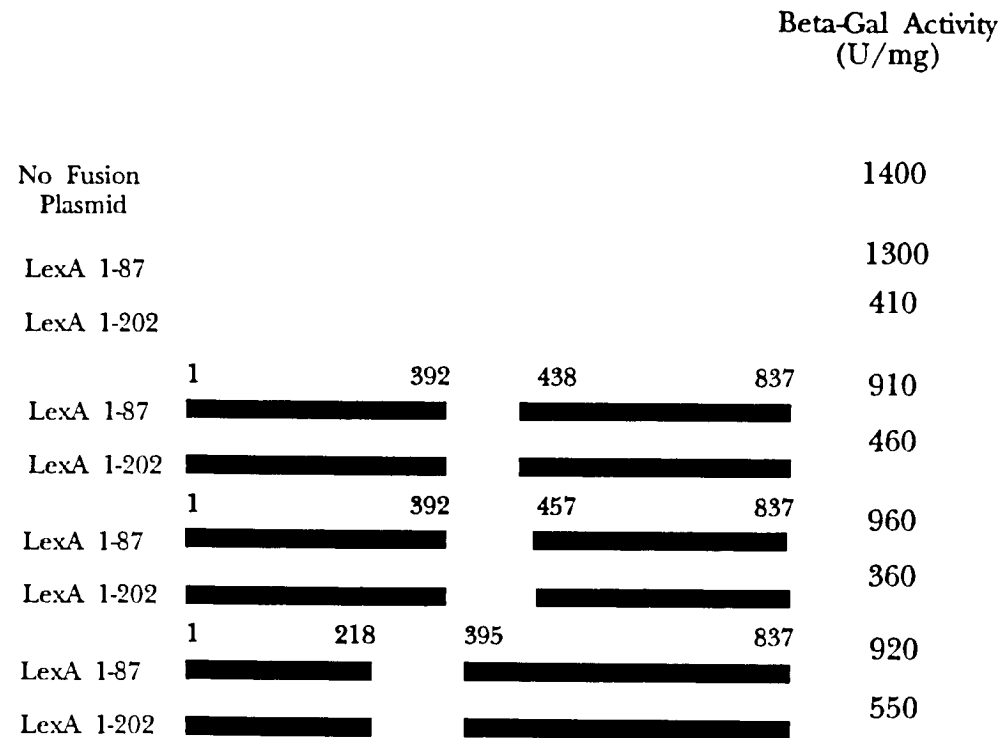
15	103	350	467	837
-----				
Q <sub>N</sub> rich region		LRR		

The solid bar represents CCR4 protein residues fused to either LexA(1-87) or LexA(1-202) as indicated. Strain 237-1b was grown in minimal medium lacking uracil and histidine and supplemented with either 8% glucose (Glucose) or 2% ethanol and 2% glycerol (Ethanol). All strains carried the 34 reporter plasmid which has 8 LexA binding sites.  $\beta$ -galactosidase units are in U/mg and represent the average of at least three separate determinations. All SEMs were less than 20% except for values less than 20 U/mg in which case SEMs were less than 30%. None of the LexA-CCR4 fusions were able to activate with the LexA reporter LR1 $\Delta$ 1 which lacks LexA operator binding sites (Figure 1 Appendix A). Western analysis indicated that all LexA-CCR4 fusions were of the expected size and of comparable abundance (data not shown). Complementation analysis was conducted in strain MD9-7c (*ccr4-10*) which displays temperature sensitive growth at 37°C on non-fermentative medium. Similar results were obtained when the ability to complement the cold-sensitivity phenotype of *ccr4-10* (growth at 16°C on glucose-containing medium) was scored.

-474), however, when fused to LexA had no ability to activate transcription (See Appendix A). The leucine-rich repeat region of CCR4 seems necessary for transcriptional activation although it does not appear to be the domain responsible for this activity. These results do not exclude the possibility, however, that deletion of the leucine-rich repeat inactivated CCR4 due to improper folding of CCR4.

In order to determine if the LexA derivatives which lacked activation potential were still capable of binding the LexA operator, a transcription interference assay was used to monitor DNA binding of the fusion proteins. The interference assay utilizes the pJK101 plasmid which carries 2 LexA operators placed between the GAL4 UAS binding site and the LacZ reporter (Appendix A Figure 1) (Brent and Ptashne, 1986). Under galactose growth conditions GAL4 binds to its UAS site and activates transcription of the reporter. Binding of LexA-fusion proteins to the LexA operator reduces this GAL4-induced transactivation. LexA fusions which were incapable of activation were examined using this assay. All three LexA(1-202)-CCR4 derivatives containing leucine-rich repeat deletions or containing only the leucine rich-repeats were capable of interfering with the GAL4 induced expression by at least two fold (Figure 4). These data indicate that their inability to activate is not due to defects in binding to the LexA operator.

Figure 4. Transcriptional Interference assays with LexA-CCR4 derivatives.



Yeast strain 237-1b containing the different LexA-CCR4 fusions and the JK101 reporter plasmid were grown in minimal medium lacking uracil and histidine and supplemented with 2% galactose and 3% glycerol. SEM values are < 20% except for those assay values under 100 U/mg whose SEM values were < 25%. None of the LexA fusion proteins affected  $\beta$ -galactosidase activity from plasmid  $\Delta$ 20B that is the same as JK101 except that it lacks the LexA binding site (Figure 1).

## Discussion

The leucine-rich repeats of CCR4 are required for CCR4 function at the ADH2 locus, for growth at elevated temperatures on non-fermentative carbon sources and for growth at cold temperatures on medium containing glucose (Malvar and Denis, 1992; See Appendix A). This region is also required by the LexA-CCR4(1-837) protein for transcriptional activity. The leucine-rich repeat alone, however, could not promote transcription. The N-terminal domains described in Appendix A may function in that role, although these regions could not complement the ccr4-10 allele *in vivo*. It is possible that deletion of the leucine-rich repeats inactivated CCR4 by placing the protein into an improperly folded form. The observation that three different leucine-rich repeat deletions each inactivated CCR4 makes this possibility seem less likely. The alternative explanation that we prefer is that the role of the leucine-rich repeats for CCR4 is to present CCR4 to its proper place at the promoter and to possibly regulate the N-terminal activation domains. The identification of two proteins by Hai-Yan Liu in the laboratory, 195 and 185 kDa in size, that form a stable complex with the leucine-rich repeats of CCR4 reinforces this notion (See Appendix A). The binding of the 195 and 185 kDa proteins to the leucine-rich repeat may make the leucine-rich repeat essential for CCR4 function.

## CHAPTER TWO

### II. IDENTIFICATION OF FACTORS ASSOCIATED WITH CCR4

#### Introduction

CCR4 is one of a group of factors distinct from the sequence specific DNA binding proteins. These intermediate factors appear to be specific to a subset of genes transcribed by RNA polymerase II. Other such factors that are like CCR4 are the SNF2,5,6, SWI 1,3 proteins, some members of the SPT family, GAL11 (Himmelfarb et al., 1990), ADA2,3 (Berger et al., 1992; Pina et al., 1993) and SUG1 (Swaffield et al., 1992). Many of these general factors appear to function as multi-subunit complexes, for example the yeast SNF/SWI complex or the SPT 4,5,6 complex (Peterson and Herskovitz, 1993; Swanson and Winston, 1992). CCR4 also appears to function as a complex of proteins. Immunoprecipitation studies have shown CCR4 to be associated with at least four other proteins a 195 kDa, 185 kDa, 146 kDa and a 116 kDa species (Draper et al., 1994). The leucine-rich repeat of CCR4 is sufficient for binding the 185 and 195 kDa proteins (Draper et al., 1994) in a high affinity manner (H. Liu unpublished observations). The SPT6, SPT10, SNF, and SWI proteins do not appear to be part of this CCR4 complex, suggesting that the mechanism by which CCR4 functions is separate from that of these other factors (Denis et al., 1994). CCR4 instead may be associated with the RNA polymerase II



holoenzyme complex since CCR4 has been shown to be specifically retained by a Pol II CTD affinity column ( R.Young and D. Chao, pers. comm.).

The core transcriptional components have been shown to be evolutionarily conserved between diverse eukaryotes. Moreover, there is evidence that the conservation of structure and function seen between the eukaryotic basic transcriptional machinery extends to the group of general factors mentioned above. Proteins with structural similarity to the yeast SNF2 protein have been identified in *Drosophila*, *Bombxyi mori*, mouse and human (Laurent and Carlson, 1992) and antibodies directed against the SWI1 and SWI3 proteins cross-react with proteins in a nuclear extract from *Drosophila* suggesting that homologs of the SWI proteins exist (Herskovitz, 1993). An SPT6 like protein, emb-5 from *C. elegans* has also been identified (Nishiwaki et al., 1993).

The pleiotropic nature of defects in CCR4 led us to believe that CCR4 and its associated factors might be found in other eukaryotes. The strong interaction between CCR4 and its 185 kDa associated protein suggested also that its specific protein contacts might be evolutionarily conserved. We have tested this hypothesis by seeking to identify proteins from mouse that interacted with CCR4. We present evidence here that the mouse mCAF1 (mouse CCR4 associated factor) protein does indeed interact and bind to the yeast CCR4 containing complex. The mCAF1 protein shares a high degree of structural similarity with proteins found in yeast, human, *C. elegans* and *Arabidopsis thaliana*. The yeast protein (yCAF1) is a CCR4 associated factor. It may be that this complex is conserved among eukaryotes and is necessary

for proper transcriptional regulation.

## Materials and Methods

### Yeast and *E. coli* strains, growth conditions, and enzyme assays.

Yeast strains are listed in Table 1. Conditions for growth of cultures on minimal medium lacking uracil and histidine or YEP medium (2% Bactopeptone, 1% yeast extract, 20 mg/liter each of adenine and uracil containing either 8% glucose or 2% ethanol as a carbon source) have been described (Cook et al., 1994). YD solid medium contained YEP supplemented to 2% glucose and 2.5% agar.  $\beta$ -galactosidase assays were conducted on yeast extracts as described (Cook et al., 1994). ADH II activity was assayed as described (Denis, 1987). Because yeast expressing LexA-CCR4 or GAL4(TA)- fusions of CCR4, yCAF1 or mCAF1 were observed to undergo loss of activity with prolonged maintenance on selective plates, assays were conducted within as short as time as possible on new transformants or freshly streaked-out colonies. Values represent the average of a least three separate determinations. Standard errors of mean were utilized in order to insure a 95% confidence level in the interpretation of the values. Transformation and genetic analysis were by standard methods (Rose et al., 1990). *E. coli* DH5 $\alpha$  was used as a host strain for all plasmids. *E. coli* were grown overnight at 37° C in L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5) containing ampicillin at a concentration of 50 ug/ml).

Table 1. Yeast Strains Used in Chapter II

Strain	<u>Genotype</u>
EGY188	<u>MATa his3 leu2 trp1 ura3 Lex<sub>OP</sub>-LEU2</u>
EGY191	<u>MAT<math>\alpha</math> his3 leu2 trp1 ura3 Lex<sub>OP</sub>-LEU2</u>
EGY191-2	<u>MAT<math>\alpha</math> his3 leu2 trp1 ura3 Lex<sub>OP</sub>-LEU2 yCAF1::LEU2</u>
MD9-7c	<u>MAT<math>\alpha</math> adh1-11 his3 trp1 ura3 ccr4-10</u>
147-6d	<u>MAT<math>\alpha</math> adh1-11 his4 leu2 trp1 ura1 spt6</u>
935-2-3	<u>MATa adh1-11 his3 leu2 trp1 ura3 yCAF1::LEU2</u>
992-6a	<u>MATa adh1-11 his3 trp1 ura3 yCAF1::LEU2</u>
994-2	<u>MAT<math>\alpha</math> adh1-11 his3 leu2 ura3 spt6::TRP1</u>

### Plasmids

LexA202 and LexA87 plasmids are 2 um plasmids and have been previously described (Brent and Ptashne, 1987; Ruden et al., 1991). The 34 reporter plasmid is a 2 um based plasmid containing eight LexA operator sites, controlling the lacZ gene (Brent and Ptashne, 1987; Cook et al., 1994). The GAL4(TA) vector (PC86) as well as the murine cDNA library in pc86, have been described elsewhere (Chevray and Nathans, 1992). The vector PJG4-5 directs the synthesis of proteins that carry at their amino termini, the influenza virus HA1 epitope tag, the B42 acidic activation domain, and the SV40 nuclear localization signal (Golemis and Brent, 1993). LexA B42 contains an E. coli derived polypeptide that activates transcription in yeast (Ruden et al., 1991).

### Plasmid Constructions

The construction of all LexA-CCR4 fusions utilized has been described elsewhere (Draper et al., 1994; Appendix A). The LexA-ADR1 fusions have been described elsewhere (Cook et al., 1993). The GAL4(TA)-yCAF1 and the LexA-yCAF1 constructs were made by placing a BamHI-HindIII fragment, where the HindIII was made blunt ended utilizing klenow fragment as described (Sambrook et al., 1989) into the BamHI-SalI site, where the SalI had been made blunt ended, of either the PC86 or LexA(202-2) vector. The resulting PC86 construct was then cut with SalI and SmaI, made blunt ended, and religated to place the coding sequence for yCAF1 in frame with the GAL4(TA). The GAL4(TA)-CCR4(1-837) fusion was made by placing an

EcoRI-BglII fragment from pTM10 containing the complete coding sequence for CCR4 into the EcoRI-BamHI site of the PC86 vector. The GAL4-cCAF1 fusion was made by placing a blunt ended EcoRV-HindIII fragment from plasmid CM21F10 containing the coding sequence for cCAF1 into the EcoRI site of PC86 made blunt ended with klenow. The HA1-B42-mCAF1 fusion was made by placing a MluI-BamHI fragment from the GAL4(TA)-mCAF1 clone, made blunt ended using Klenow, into the filled in EcoRI site of PJG4-5.

#### Native immunoprecipitations

Cells were grown in 2 liters of either YEP or an appropriate selective medium containing 8% glucose or 3% ethanol as described above. Cells were harvested at a density of  $5 \times 10^7$  by centrifugation, were washed in 20 ml of ice cold water, and were resuspended in two volumes of native extract buffer (25 mM  $\text{KPO}_4$  buffer, pH 7.6/150 mM KCl/1 mM NaPPi/1 mM NaF/5 mM  $\text{MgCl}_2$ / 1 mM EDTA/10% Glycerol/1% NP-40/8  $\mu\text{M}$  Leupeptin/3  $\mu\text{M}$  Pepstatin A/1 mM PMSF) prior to lysis in a bead beater according to manufacturers specifications. The resulting lysates were spun in a microfuge for 15 minutes at maximum speed. The supernatant fluid was then mixed with pre-immune sera coupled to protein A agarose (or Protein A agarose alone) at a 2:1 ratio and incubated at 4°C for 15 minutes with gentle rocking. After clarification in a clinical centrifuge the pre-cleared extract was mixed with anti-sera coupled to protein A agarose at a 5:1 ratio. The immune beads and the supernatant were incubated for 45 minutes at 4°C with rocking. Immune beads were washed

three times with 10 volumes of ice cold native extract buffer and twice in 10 volumes of ice cold triton wash buffer (20 mM NaPO<sub>4</sub> buffer, pH 7.6/140 mM NaCl/1 mM EDTA/0.5% Triton X-100). Western blotting was performed with purified polyclonal rabbit antisera raised against whole LexA protein or peptides based on the N-terminus of CCR4, the N-terminus of yCAF1(SQRQASEQHQQQNMGPQCC), or the C-terminus of yCAF1(CCKYQGVIIYGIDGDQ). Antibody against GAL4 was a gift from Dr. Jim Hopper. Western or silver staining was performed as described (Vallari, et al., 1992; Wray et al., 1981).

#### Isolation of clones, Sequencing, and Protein sequence analysis

The full length yCAF1 gene was isolated from a yeast YCp-50 genomic library by colony hybridization (Sambrook et al., 1989). The full length cCAF1 clone was obtained from R.H. Waterson, Washington University School of Medicine. Insert DNA containing mCAF1 and cCAF1 were sequenced on each strand by double stranded sequencing with deoxynucleotides and Sequenase (US Biochemical). Sequence comparison analysis was performed at the National Center for Biotechnology Information using the BLAST network service (Altschul et al., 1990). Alignments were performed using the clustal method available in the megalign portion of the DNA STAR package (DNA STAR Inc.).

## Results

### **LexA-CCR4 is capable of interacting with a protein from mouse**

We used a yeast genetic selection scheme, the two-hybrid system or the interaction trap, to isolate and characterize proteins from mouse which were capable of interacting with CCR4. In this technique a library containing conditionally expressed cDNA-encoded proteins from mouse fused to the GAL4 trans-activation domain (GAL4(TA)) was transformed into a strain of yeast containing a plasmid directing the synthesis of a LexA(1-202)-CCR4(1-837) fusion and two different reporters. One reporter, LexAop-LEU2, allowed growth in the absence of leucine when activated. The second LexAop-LacZ directed the synthesis of beta-galactosidase, under the control of LexA. LexA-CCR4 under glucose growth conditions activated the LexAop-LEU2 and LexAop-LacZ reporters only weakly (Draper et al., 1994). We screened  $1 \times 10^6$  primary library transformants for yeast which exhibited a strong Leu<sup>+</sup> phenotype and turned a dark blue color when placed on X-gal indicator plates. Only two isolates showed dependence on both the LexA-CCR4 plasmid and the GAL4-mouse cDNA fusion for growth on leucine and for increased beta-galactosidase activity. Restriction analysis and sequencing showed that both positive clones were identical. We named this gene mCAF1 (mouse CCR4 associated factor). Antibody to the GAL4 protein specifically identified the GAL4-mCAF1 protein in yeast crude extracts at the 50 kDa size predicted from the sequencing of the GAL4-mCAF1 plasmid (data not shown). The GAL4(TA)-mCAF1

protein did not bind to LexA alone or activate transcription by itself since strains carrying just LexA with either the GAL4(TA) or the GAL4(TA)-mCAF1 fusion failed to exhibit increased  $\beta$ -galactosidase activity (Figure 5). Also, the GAL4 moiety was not binding to CCR4 as strains containing LexA-CCR4 with either GAL4(TA) or GAL4(TA)-CCR4 failed to show increased activity. Placement of another LexA fusion, LexA-yTFIIB with both GAL4(TA) and GAL4(TA)-mCAF1 also failed to exhibit any increase in activity over just LexA-yTFIIB alone (Figure 5). Only LexA-CCR4 with the GAL4(TA)-mCAF1 fusion gave increased  $\beta$ -galactosidase activity, indicating a specific interaction between CCR4 and mCAF1 (Figure 5). To further verify the specificity of this interaction we fused the mCAF1 open reading frame to the coding sequence for a different activator HA1-B42. B42 is an *E.coli* derived sequence which activates transcription in yeast when bound to DNA. This HA1-B42-mCAF1 fusion construct along with LexA-CCR4 resulted in increased  $\beta$ -galactosidase activity over just the HA1-B42 containing vector alone with LexA-CCR4 (Figure 5).

#### **LexA-CCR4 co-immunoprecipitates GAL4(TA)-mCAF1.**

To examine whether there was a true biochemical interaction between CCR4 and mCAF1 co-immunoprecipitation experiments were conducted. When antibody directed against LexA was used to immunoprecipitate LexA-CCR4 from a strain expressing the GAL4(TA)-mCAF1 fusion, a band migrating at 50 kDa corresponding



<u>LexA-Fusion</u>	<u>GAL4(TA) Fusion</u>	<u>Beta-Gal Activity (mU/mg)</u>
LexA	GAL4(TA)	4.4
LexA CCR4 (1-837)	GAL4(TA)	15
LexA CCR4 (1-837)	GAL4(TA) mCAF1	560
LexA	GAL4(TA) mCAF1	2.8
LexA TFIIIB(1-345)	GAL4(TA) mCAF1	11
LexA TFIIIB(1-345)	GAL4(TA)	10
LexA CCR4 (1-837)	GAL4(TA) CCR4 (1-837)	15
LexA CCR4 (1-837)	HA1-B42 mCAF1	350
LexA CCR4 (1-837)	HA1-B42	16

Figure 5. Interaction of mCAF1 with CCR4. Plasmids that directed the synthesis of various activator tagged proteins were introduced into strain EGY188 along with the various baits listed. The 34 reporter containing 8 LexA binding sites was used in all cases. Activation was monitored by  $\beta$ -galactosidase assay in liquid cultures grown on 8% glucose except for those strains with HA1-B42 tagged proteins which were grown in 2% galactose and 2% raffinose to induce the expression of the HA1 tagged proteins.  $\beta$ -galactosidase assays were performed at least in triplicate. All SEMs were less than 20%.

to GAL4-mCAF1 was specifically detected by Western analysis using the GAL4 antibody (Figure 6, lane a). A similar result was obtained from a strain carrying a LexA(1-87)-CCR4 fusion and GAL4-mCAF1 (data not shown). The 50 kDa band corresponding to GAL4(TA)-mCAF1 was absent when immunoprecipitations were conducted with extracts from a strain lacking the GAL4(TA)-mCAF1 fusion, confirming that this 50 kDa protein was the GAL4(TA)-mCAF1 fusion (Figure 6, lane b). The LexA antibody also failed to co-immunoprecipitate the GAL4(TA)mCAF1 fusion from extracts of a strain containing LexA alone and GAL4(TA)-mCAF1 (Figure 6, lane d). Immunoprecipitation of LexA-CCR4 from a strain containing the GAL4(TA)-CCR4 fusion failed to show an interaction in the co-immunoprecipitation experiment, indicating the interaction was not between GAL4(TA) and LexA-CCR4 (Figure 6, lane e). Utilizing an antibody raised against a CCR4 N-terminal peptide, we immunoprecipitated wild type CCR4 from a strain containing a GAL4(TA)-mCAF1 fusion. Immunoprecipitation of wild type CCR4 also brought down the 50 kDa mCAF1 fusion species (Figure 6 lane f). This band was not detected when a strain without the GAL4(TA)-mCAF1 fusion was utilized (data not shown). These data confirm that the interaction observed between CCR4 and mCAF1 using the two-hybrid system results from an *in vivo* physical association of CCR4 with mCAF1.

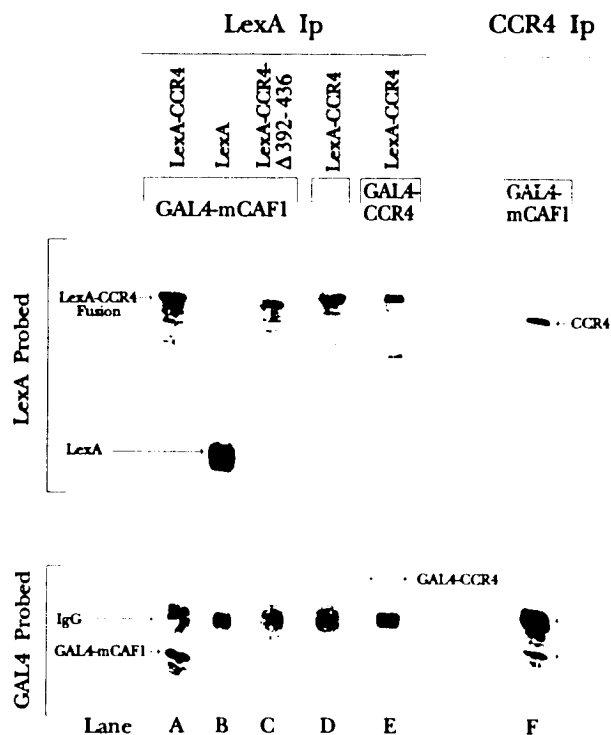


Figure 6. mCAF1 co-immunoprecipitates with CCR4. Native immunoprecipitations were conducted as described in materials and methods from strains grown in 8% glucose. Antibody against LexA or CCR4 was used to immunoprecipitate the LexA-CCR4 fusions (LexA Ip) or wild type CCR4 (CCR4 Ip) respectively. Samples were subsequently subjected to SDS page and Western analysis. Western blots were probed with GAL4 antibody to detect presence of the GAL4(AD)-mCAF1 fusion (GAL4 probed), LexA antibody to detect the presence of LexA or LexA-fusions (LexA probed), or CCR4 antibody to detect the presence of CCR4. Lanes A,B, and C are LexA immunoprecipitates from strains expressing LexA-CCR4, LexA, or LexA-CCR4 $\Delta$ 392-436 respectively along with GAL4(TA)-mCAF1. Lane D- LexA-CCR4 immunoprecipitated from a strain expressing no GAL4(TA) fusion. Lane E- LexA-CCR4 immunoprecipitated from a strain expressing a GAL4(TA)-CCR4 fusion. Lane F- CCR4 immunoprecipitated from a strain expressing the GAL4(TA)-mCAF1 fusion.

**mCAF1 is structurally similar to proteins in human, *C.elegans*, *Arabidopsis* and yeast**

Sequencing of the mCAF1 reading frame from the GAL4-mCAF1 containing plasmid revealed, as expected, that it was fused in frame to the invariant amino-terminal moiety of the vector encoded GAL4(TA). Although mCAF1 contained about 1.2 kb of coding sequence it lacked the translational initiation codon. We used this cDNA fragment to isolate a larger 1.2 kb cDNA from a mouse 3T3-L1 adipocyte cDNA library. The sequence from this clone revealed a complete open reading frame of 285 amino acids that was identical to the original mCAF1 gene except for an N-terminal extension of 15 amino acids (Figure 7). Database searches utilizing the amino acid sequence for mCAF1 led to the identification of four proteins with a high degree of similarity from human, *C. elegans*, *Arabidopsis* and yeast. The *C.elegans* protein, which we called cCAF1 (C.elegans CCR4 associated factor), the human protein, hCAF1 and the *Arabidopsis thaliana*, aCAF1 were all found submitted in the dbest database as partial cDNA sequences. The yeast gene, yCAF1 was found initially in genbank as a partial open reading frame within a larger cloned fragment and in later searches as a complete sequence under the name POP2. POP2 had been identified as being a transcription factor involved in the regulation of the yeast PGK, SUC2 and ICL1 genes involved in glycolysis, sucrose metabolism and the glyoxylate cycle respectively.

The mCAF1 and hCAF1 sequences displayed 99% identity at the DNA level over the 190 nucleotides of the hCAF1 sequence available from the dbest database. Due to

this near identity the hCAF1 sequence was not further analyzed. Instead, in order to make a broader evolutionary comparison, the cCAF1 cDNA was obtained, sequenced in its entirety, and shown to encode a protein of 310 amino acids. Examination of the yCAF1 protein revealed 40% identity and 60% similarity at the amino acid level with the mCAF1 protein and 30% identity and 56% similarity with the cCAF1 protein. The cCAF1 protein was 48% identical to mCAF1. The strongest region of identity between these three proteins was between residues 174 to 341 of the yeast protein in which there is 32% identity between cCAF1, mCAF1 and yCAF1 (Figure 7). The yCAF1 protein differs from the mCAF1 and cCAF1 proteins in that it contains an N-terminal extension of 148 residues. The aCAF1 sequence was submitted to the dbest database while this manuscript was in preparation and was not analyzed further. Besides the similarity of the CAF1 proteins to each other they share no other similarities to other proteins in the database.

#### **Both yCAF1 and cCAF1 interact with CCR4**

We inserted the yCAF1 coding sequence in the GAL4(TA) vector to address whether yCAF1 could interact with CCR4. The GAL4(TA)-yCAF1 fusion gave a positive result in the interaction trap with LexA-CCR4 (Figure 8). Further, in screening for yeast proteins which would associate with CCR4, using a yeast expression library consisting of yeast genomic DNA fused to the B42 activation domain, we obtained several yCAF1 clones (data not shown). The partial amino acid

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1  - - - - - M P A A T V D H S Q R I C E V W A C N Mouse
1  M A S S S S G G A G G A S G A P E V K I H N Y Y M S N Nematode
149 L P K H L T N Q S M P P I F L P P P N Y L F V R D V W K S N Yeast
1  - - - - - P P N P E E E E D D T I E I R E V W N H N Arabidopsis

20 L D E E M K K I R Q V I R K Y N Y V A M D T E F P G V V A R Mouse
31 V E E E F A R I R G F V E D Y P Y V A M D T E F P G V V A T Nematode
179 L Y S E F A V I R Q L V S Q Y N H V S I S T E F V G T L A R Yeast
21 L E Q E M A L I E Q S I D D F P Y V A M D T E F P G I V C K Arabidopsis

50 P I G E - - - - - F R S N A D Y Q Y C L L R C N V D L L K Mouse
61 P L I G T - - - - - F R S K E D F N Y C Q V F C N V N M L K Nematode
209 P I G T - - - - - F R S K V D Y H Y Q T M R A N V D F L N Yeast
51 T V T A N P N P N P Y S I H Y E Y N Y D T L K A A V N M L K Arabidopsis

74 I I Q L G E T F M N E Q G E Y P P G T S - - - T W Q F N F Mouse
85 L I Q V G F A M V N D K G E L P P T G D - - - V W Q F N F Nematode
233 P I Q G L S L S D A N G N K P D N G P S - - - T W Q F N F Yeast
81 L I Q G L T L S D E K G N L P T C G T N K Q C I W Q F N F Arabidopsis

100 K F N L T E D M Y A Q D S I E L L T T S G I Q F K K H E E E Mouse
111 N F S F A E D M F S H E S V E M L R Q A G I D P T L L Q N N Nematode
260 E F D P K K E I M S T E S L E L L R K S G I N F E K H E N S Yeast
111 R Arabidopsis

130 G I E T R Y F A E L L M T S G V V L C E G V K W L S F H S G Mouse
141 G I P T A V P G E L L T T S G L I T D P R I T W L T F S S G Nematode
290 G I D V F E F S Q L L M D S G L M M D D S V T W I T Y H A A Yeast
111 Arabidopsis

160 Y D F G Y L I K I L T N S N L P E E E L D F F E I L R L F F Mouse
171 Y D F G Y L K S I T L G D L P K E E S T F F M C H K T L F Nematode
320 Y D L G F L T N I L M N D S M P N N K E D F E W W V H Q Y M Yeast
111 Arabidopsis

190 P V I Y D V K Y L M K S - - - C K N - - L K G G - - - - - Mouse
201 P T S F D I R I L L R T - P N C X S A K L K G G - - - - - Nematode
350 P N F Y D L N L V Y K I I Q E F K N P Q L Q Q S S Q Q Q Q Q Yeast
111 Arabidopsis

209 - - - L Q E V A E Q L E L E R I G P Q H Q A G S D S L L T Mouse
224 - - - L Q E V A D Q L D V K R Q G V R H Q A G S D A L L T Nematode
380 Q Q Y S L T T L A D E L G L P R F S I F T T T G G Q S L L M Yeast
111 Arabidopsis

225 G M A F F K M R E M F F E D H I D D A K - - Y C G H L Y G L Mouse
250 A A T F F K I K K Q F F G D N W N Q I A P L I C G H M F G L Nematode
410 L L S F C Q L S K L S M - - - - - - - - - H K F P N Yeast
111 Arabidopsis

263 G S G S S - V V Q N G T G N A Y E E E A S K - - - - - Mouse
280 G S S L S L F H S S G S T S R L G D E T P Q G L I - G V P Q Nematode
427 G T D F A K Y - - - - - - - - - - Q G V I Y G I D G Yeast
111 Arabidopsis

284 Q S Mouse
309 Q A Nematode
443 D Q Yeast
111 Arabidopsis

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Figure 7. Alignment of the CAF1 proteins from yeast, mouse, *C.elegans* and *Arabidopsis*. The alignment was obtained with the aid of the MEGALIGN program from the DNA STAR computer package (DNA STAR Inc.). Residues sharing identity have been boxed and shaded.

sequences of these yCAF1 fragments that interacted with CCR4 completely overlapped with the region of similarity between yCAF1 and mCAF1. We consequently examined whether cCAF1 could interact with CCR4 by constructing a GAL4(TA)-cCAF1 fusion. A significant, albeit weak, interaction between cCAF1 and CCR4 was observed (Figure 8). This weak interaction is most likely due to the decreased abundance of cCAF1 in yeast since Western analysis indicated that GAL4-cCAF1 expression was at least ten-fold less than that observed for GAL4(TA)-mCAF1 or GAL4(TA)-yCAF1 (data not shown).

**The leucine rich repeat of CCR4 is essential for the interaction of yCAF1 and mCAF1 with CCR4.**

In order to localize the domain responsible for the interaction of CCR4 with yCAF1 and mCAF1 we utilized various LexA-CCR4 derivatives in the interaction trap. As seen in Figure 8 deletion of the N-terminus (LexA-CCR4  $\Delta$ 14-208) or truncation of the CCR4 (LexA-CCR4-1-668) had only small effects on the interaction of yCAF1 and mCAF1 with CCR4 as judged by the increase in  $\beta$ -galactosidase activity over the corresponding LexA-CCR4 fusion with just GAL4(TA). However, small deletions within the leucine rich repeat,  $\Delta$ 391-435,  $\Delta$ 391-455,  $\Delta$ 218-394, completely abolished the interaction of yCAF1 and mCAF1 with CCR4. In support of the conclusion that the leucine rich-repeat of CCR4 is a domain important for the interaction of mCAF1 and yCAF1 with CCR4, immunoprecipitation of a strain

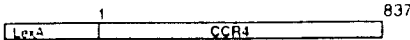
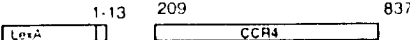
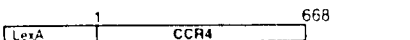
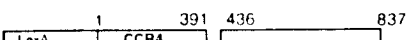
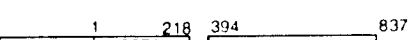

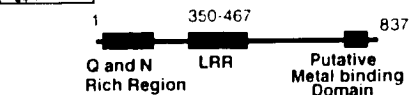
LexA(1-202)-CCR4 Fusion		No GAL4TA Fusion	GAL4TA-mCAF1	GAL4TA-yCAF1	GAL4TA-cCAF1
		$\beta$ -Gal (mu/mg)	$\beta$ -Gal (mu/mg)	$\beta$ -Gal (mu/mg)	$\beta$ -Gal (mu/mg)
		15	560	300	52
		15	290	60	ND
		<2	60	25	ND
		<2	<2	<2	ND
		<2	<2	<2	ND
		<2	<2	<2	ND
					

Figure 8. Identification of the domain in CCR4 responsible for the interaction with CAF1. Various LexA-CCR4 derivatives were placed with GAL4(TA) fusions of mCAF1, yCAF1 and cCAF1. All strains carried the 34 reporter which has 8 LexA binding sites. Due to the weak nature of the interaction between CCR4 and cCAF1 we placed cCAF1 only with the LexA-CCR4(1-837) fusion.  $\beta$ -galactosidase assays are in U/mg and represent the average of at least three separate determinations. All SEMs were less than 20%.



carrying LexA-CCR4( $\Delta$ 391-435) and GAL4-mCAF1 failed to bring down the mCAF1 protein (Figure 6 lane c). However, we were not able to demonstrate that the leucine-rich region alone could interact with yCAF1 or mCAF1. Taken together, these data support the conclusion that the leucine rich-repeat of CCR4 may be necessary but not sufficient for the association of mCAF1 and yCAF1 with CCR4.

### **yCAF1, CCR4 and the CCR4 associated 185 and 195 kDa proteins form a complex**

Previous work had shown that CCR4 is complexed with at least two other proteins, 185 and 195 kDa in size. We conducted co-immunoprecipitation experiments to determine if yCAF1 was also complexed with CCR4 as well as the 185 and 195 kDa proteins. Antibody directed against either LexA or CCR4 was used to immunoprecipitate native extracts of strains containing either LexA-yCAF1(1-444) or just LexA. Immunoprecipitates were subsequently subjected to SDS PAGE and then silver staining or Western analysis. When antibody directed against LexA was utilized in the immunoprecipitations a band corresponding to the predicted size of the LexA-yCAF fusion was seen by silver staining (data not shown). This band was subsequently shown to be the LexA fusion by Western analysis using an anti-LexA antibody (Figure 9 lane b) and was absent from a strain containing just LexA alone (Figure 9 lane a). Also visible by silver staining was a band corresponding to the size of CCR4 (data not shown). We confirmed that this protein was CCR4 by probing a blot with an anti-CCR4 antibody (Figure 9 lane b). We detected no CCR4 in a strain

where just LexA alone had been immunoprecipitated (Figure 9 lane a). Further, when CCR4 antibody was used to conduct the immunoprecipitation LexA-yCAF1 was specifically immunoprecipitated (Figure 9 lane c). No protein band corresponding to LexA-yCAF1 was observed in CCR4 immunoprecipitations from a strain containing just LexA (Figure 9 lane d). Not only did CCR4 and LexA-yCAF1 co-immunoprecipitate but immunoprecipitating LexA-yCAF1 with LexA antibody also revealed the presence of the 185 and 195 kDa CCR4 associated species (Figure 10 lane a). These bands were not immunoprecipitable from a strain containing just LexA alone (Figure 10 lane b).

We next examined whether CCR4 was necessary for the interaction of yCAF1 with the 185 and 195 kDa proteins. Immunoprecipitations from a strain lacking CCR4 (MD9-7c) utilizing anti-LexA antibody brought down both the 185 and 195 kDa proteins (Figure 10, lane c). Further, immunoprecipitation of CCR4 in a strain containing a ycaf1 disruption revealed that CCR4 was still able to associate with the 185 protein (figure 10, lanes d and e), although, the 195 kDa protein was not visible. These data indicate that the 185 kDa protein binds independently to CCR4 and yCAF1. We also noted a decreased yield in the amount of CCR4 and 185 protein that we could immunoprecipitate from strains lacking yCAF1 (data not shown). Because the abundance of CCR4 in non-immunoprecipitated extracts from a strain lacking CAF1 was not affected (data not shown), it appears that the CAF1 protein is required for efficient CCR4 immunoprecipitation.

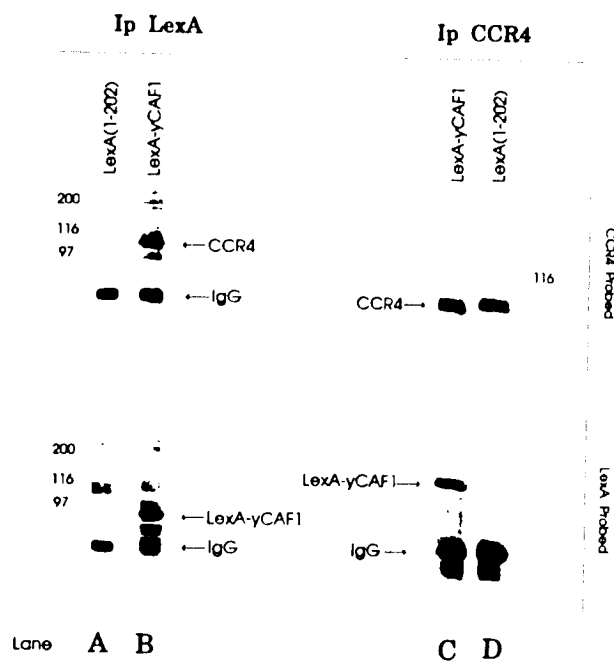


Figure 9. yCAF1 specifically interacts with CCR4 as judged by immunoprecipitation. Conditions for immunoprecipitations are described in Materials and Methods. Lanes A and B- LexA antibody was used to conduct the immunoprecipitations (Ip LexA). Lanes C and D- CCR4 antibody was used to conduct the immunoprecipitations (Ip CCR4). Proteins were visualized by Western analysis with either antibody against CCR4 (CCR4 probed) or LexA (LexA probed).

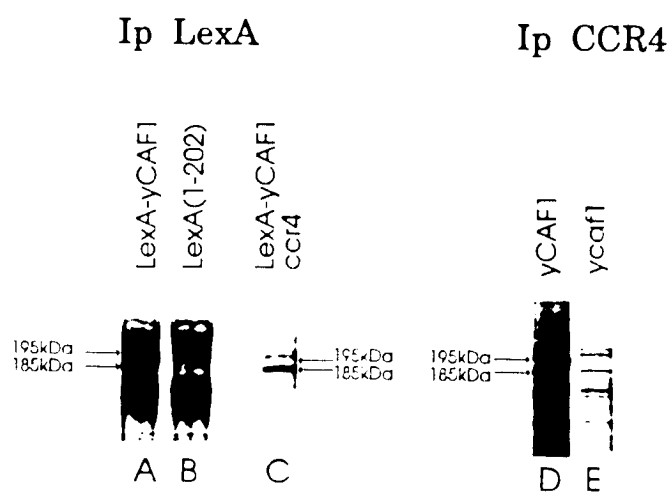


Figure 10. yCAF1 is complexed with the 185 and 195 kDa proteins. Native immune precipitations were conducted as described in Material and Methods. The 185 and 195 kDa proteins are indicated with arrows. Lanes A and B- immunoprecipitation of LexA-yCAF1 or LexA respectively from strain EGY188 utilizing antibody against LexA. Lane C- immunoprecipitation of LexA-yCAF1, using antibody against LexA, from strain MD9-7c (*ccr4-10*) which lacks *ccr4*. Lanes D and E-immunoprecipitation of CCR4, using antibody against CCR4, from strain EGY191 (*yCAF1*) and strain EGY191-2 (*ycaf1*) respectively.

### **Wild type yCAF1 is associated with CCR4 in vivo**

While the above results indicated that LexA-yCAF1 physically interacted with CCR4 we wished to also establish that the yeast CAF1 protein at its normal physiological concentration was bound to CCR4. Immunoprecipitation of CCR4 utilizing an antibody against CCR4 and subsequent probing of the resulting blots with the yCAF1 antibody, raised against an N-terminal peptide of yCAF1, revealed a 50 kDa band running just under the IgG (Figure 11, lane a). This 50 kDa band, which is the expected size of yCAF1, was also identified with yCAF1 antibody raised against a C-terminal peptide of yCAF1 (Figure 11, lane b) and was absent in a strain lacking yCAF1 confirming its identity as CAF1 (data not shown). Moreover, the anti-CAF1 antibodies specifically recognized a LexA-yCAF1 and a MBP-yCAF1 fusion (data not shown). In conducting the reverse experiment by immunoprecipitating with antibody against the N-terminus of yCAF1 and subsequent probing of the resulting blot with the CCR4 antibody, CCR4 was found to co-immunoprecipitate with yCAF1 (Figure 11, lane c). These results confirm that CAF1 is physically associated with CCR4.

### **Disruption of the yCAF1 gene in yeast gives phenotypes similar to disruptions of CCR4**

In order to examine the functional association of CAF1 with CCR4 a disruption of the CAF1 gene in yeast was made. Disruption of CAF1 in strain EGY191 resulted in a temperature sensitive phenotype on YPD media at 37°C (data not shown). Previous

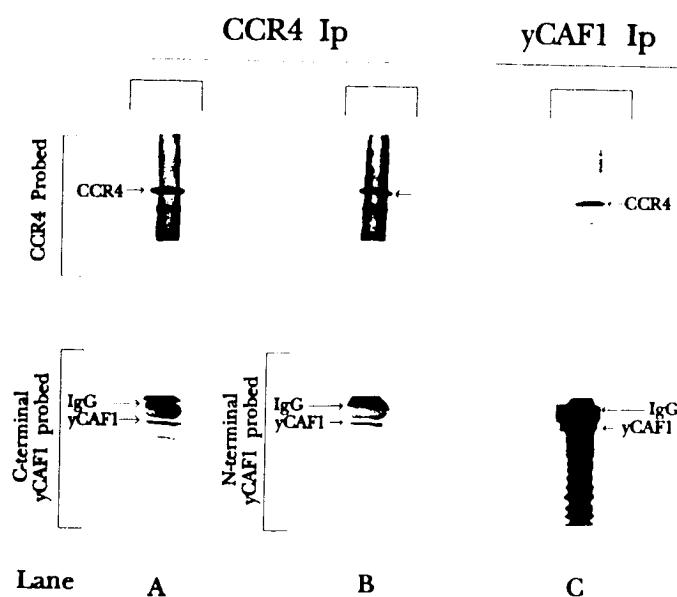


Figure 11. Wild type yCAF1 is associated with CCR4. Conditions for immunoprecipitations and Western blotting are described in Materials and Methods. Lanes A and B- CCR4 antibody was used to conduct the immunoprecipitations (CCR4 Ip). Lane C- yCAF1 antibody directed against an N-terminal peptide of yCAF1 was used to conduct the immunoprecipitation. Proteins were visualized by western analysis with either CCR4 antibody (CCR4 probed) or yCAF1 antibody (C-terminal, Lane A, or N-terminal, Lanes B and C, yCAF1 probed).

analysis of the POP2 (CAF1) gene in yeast identified this temperature sensitive phenotype as well as a general defect for growth in complete synthetic medium containing ethanol or glycerol as the carbon source (Sakai et al., 1993). This phenotypic defect for growth on non-fermentative medium is similar to that observed with ccr4 disruptions which exhibit temperature sensitivity at 37°C when utilizing a non-fermentative carbon source. A caf1 strain (935-2-3) also displayed a two-fold lower ADH II activity under derepressed conditions as compared to an isogenic strain differing only in the CAF1 allele (935-2) (Table 2A). This phenotype is similar to that of a ccr4 defect. In a strain carrying both a caf1 and ccr4-10 allele (935-1-6) a further two-fold decrease in ADH2 expression was observed as compared to an isogenic ccr4-10 containing strain (935-1) (Table 2A).

CCR4 is the only known gene to be epistatic to the spt6 and spt10 alleles. The spt6 and spt10 alleles cause a partial release of ADH2 from the repressed state seen on glucose. Mutations in CCR4 cause a decrease in the ADH II activity under glucose growth conditions seen in an spt6 or spt10 genetic background. This fact led us to examine whether yCAF1 was also epistatic to the SPT6 and SPT10 genes. Disruption of CAF1 suppressed the increased ADH2 expression under glucose growth conditions that is observed in an spt6 and spt10 mutant background (Table 2B). The similarity of these phenotypes observed with a caf1 disruption to that of a ccr4 defect supports the conclusion that CAF1 is a functional component of the yeast CCR4 transcriptional regulatory complex.

A		
Strain	Genotype	ADH II Activity (mU/mg) 2% Ethanol
935-2	yCAF1	1500
935-2-3	ycaf1	760
935-1	yCAF1 ccr4-10	420
935-1-6	ycaf1 ccr4-10	160

B		
Strain	Genotype	ADH II Activity (mU/mg) 8% Glucose
Segregants <sup>a</sup>	spt6	60
Segregants <sup>a</sup>	spt6 ycaf1	6.2
Segregants <sup>b</sup>	spt10	44
Segregants <sup>b</sup>	spt10 ycaf1	1.6

Table 2. Phenotypes of a yCAF1 disruption. ADH II activities were measured as described in Materials and Methods. All values represent the average of at least three determinations or in the case of segregants the average of at least four different strains.

<sup>a</sup>Segregants from cross 147-6d X 935-2-3

<sup>b</sup>Segregants from cross 992-9a X 994-2



### **yCAF1 is necessary for full activation by LexA hybrid activators**

We have previously shown that the CCR4 gene is required for maximal transcriptional activation by several LexA bound trans-activator proteins (Draper et al., 1994; unpublished observation). We therefore examined whether disruptions of CAF1 could also affect the ability of LexA bound activators to function in yeast and whether transcriptional activation by LexA-CCR4 was CAF1 dependent. The results shown in table 3 indicate that the caf1 allele resulted in a 2 to 5 fold reduction in the activation potential of each of the activators tested. This reduction occurred for weak activators like full-length CCR4 or TADII from ADR1 and was observed for stronger activators like B42 and the individual CCR4 activation domains which exhibit strong activation potential when removed from the CCR4 C-terminal sequences (Draper et al., 1994; Appendix A). These results are similar to what had previously been observed with disruptions of CCR4 (Draper et al., 1994). We interpret this data to mean that yCAF1, like CCR4, is important for full activation potential but does not appear to be essential to any one particular type of activator.

### **LexA-yCAF1 can activate transcription when fused to LexA**

The ability of yCAF1 to function as a transcriptional activator when bound to LexA was examined by assaying the ability of a LexA-yCAF1(1-444) fusion to activate a LexA operator controlled  $\beta$ -galactosidase gene. LexA-yCAF1(1-144) functioned as a strong transactivator protein (Figure 12). Unlike CCR4 though the activation potential showed no carbon source regulation (Draper et al., 1994).

LexA-Fusion Protein	yCAF1 Genotype	$\beta$ -Galactosidase Activity(U/mg)	Fold Decrease in Activation
LexA(1-202)-CCR4(1-13/210-344)	<u>yCAF1</u>	2400	2.5
	<u>ycaf1</u>	960	
LexA(1-202)-CCR4 (1-159)	<u>yCAF1</u>	1000	4.3
	<u>ycaf1</u>	230	
LexA(1-202)-CCR4(1-837)	<u>yCAF1</u>	15	3.3
	<u>ycaf1</u>	4.6	
LexA(1-202)-ADR1(147-358)	<u>yCAF1</u>	150	4.7
	<u>ycaf1</u>	32	
LexA(1-87)-B42	<u>yCAF1</u>	960	3.4
	<u>ycaf1</u>	280	

Table 3. Mutations in yCAF1 affect the activation potential of various activators.  $\beta$ -galactosidase activities were conducted as described. The 34 reporter was used in all cases. All SEMs were less than 20%. Strain EGY191 (yCAF1) and EGY191-2 (ycaf1) are isogenic except for the yCAF1 allele.

LexA fusions of either mCAF1 or cCAF1 lacked the ability to function as activators (Figure 12). A LexA-yCAF1 fusion (amino acids 182-444) which encompassed the regions sharing similarity with the mouse and *C. elegans* also failed to show any activation potential (Figure 12). Further, we were unable to demonstrate a positive result for interaction with a GAL4(TA)-CCR4(1-837) utilizing LexA fusions of mCAF1, cCAF1 or yCAF1 (182-444) in the two hybrid system (Figure 12). We interpret this to mean that the LexA-domain is interfering with some aspect of the interaction, as longer LexA fusions of yCAF1 (1-444 or 88-444) all function and as indicated earlier, GAL4 and HA fusions of both cCAF1 and mCAF1 are functional (Figure 8). We next tested whether the N-terminal region of yCAF1 (amino acids 1-181) could function as an activator when fused to LexA. This fusion was unable to demonstrate any activation potential and showed no interaction with GAL4(TA)-CCR4 (Figure 12). We believe that the ability of yCAF1 to activate may be dependent upon association with the CCR4 complex. However, activation by the LexA(1-202)-yCAF1 fusion was only affected two fold in a strain lacking CCR4 (data not shown), indicating that while CCR4 function is necessary for full activation by yCAF1 it is not essential. CCR4 may just be one component of the complex that is involved in providing the LexA-yCAF1 fusion with activation potential.

		Beta-Gal Activity		Interaction with GAL4(TA)-CCR4
		Glucose	Ethanol	
LexA 1-202		<1.5	<1.5	-
LexA(1-202)	<div> <div>1</div> <div>444</div> <div>yCAF1</div> </div>	360	400	+
LexA 1-202	<div> <div>182</div> <div>444</div> <div>yCAF1</div> </div>	3.9	2.6	-
LexA 1-202	<div> <div>1</div> <div>181</div> <div>yCAF1</div> </div>	<1.5	<1.5	-
LexA 1-202	<div> <div>1</div> <div>285</div> <div>mCAF1</div> </div>	<1.5	<1.5	-
LexA 1-202	<div> <div>1</div> <div>310</div> <div>cCAF1</div> </div>	<1.5	<1.5	-

Figure 12. LexA-yCAF1 can function as an activator.  $\beta$ -galactosidase assays were conducted as described in Materials and Methods. Interaction with GAL4(TA)-CCR4 was judged based on increased  $\beta$ -galactosidase values on glucose.

## Discussion

### **CAF1 is an evolutionarily conserved protein that associates with CCR4**

We have identified a protein from mouse which is capable of interacting and binding to the yeast CCR4 regulatory complex. The mCAF1 protein was shown to have a high degree of structural similarity to proteins from human, *C.elegans*, *Arabidopsis* and yeast. Since the CAF1 sequences are derived from different tissues at varied developmental stages and from vastly different eukaryotic organisms we believe that the CAF1 protein is an important regulatory component ubiquitous to all cell types and most if not all eukaryotic organisms. The yCAF1 protein contained an extra 144 amino acids at the N-terminal region lacking from the other homologs. This region while not required for binding CCR4 was important for function of the yCAF1 protein specifically in terms of complementing a caf1 allele. The observation that mCAF1, cCAF1 and the C-terminal region of yCAF1 each bind to or interact with CCR4 indicates that this segment of CAF1 has retained an evolutionarily conserved function. It is likely therefore that CCR4 homologs or complexes exist in higher eukaryotes.

Immunoprecipitation experiments revealed that yCAF1 was complexed with the 185 and 195 kDa proteins previously shown to be associated with CCR4. Further, the binding of CCR4 and yCAF1 to the 185 protein was not interdependent. It is possible that the 185 and 195 kDa proteins, which are possibly modified forms of the same protein, act as a scaffolding upon which CCR4 and CAF1 are bound.

Alternatively, CAF1, CCR4, 185 and 195 kDa proteins could be engaged in a trimolecular interaction. The stoichiometry of these proteins would indicate that they interact in an equimolar ratio. Although a *caf1* disruption significantly reduced the amount of CCR4 and 185 kDa protein that was immunoprecipitable from a yeast extract (Figure 10), the small amount of CCR4 and 185 kDa protein that was co-immunoprecipitable still was in a 1:1 ratio (Figure 10). These results indicate that CAF1 is required for the accessibility of this complex by antibody which recognizes the N-terminal sequence of CCR4. It is likely that CAF1 association with CCR4 alters the configuration of the CCR4 complex.

#### **CAF1 is required for diverse transcriptional processes**

Disruption of *yCAF1* gave phenotypes similar to those seen with disruptions of *CCR4*. A *caf1* allele affected the ability of the *ADH2* gene to derepress, eliminated the increased ADH II activity observed with mutations in *SPT6* or *SPT10* and caused a general defect in the ability to utilize non-fermentative carbon sources. Moreover, a *caf1* disruption resulted in a temperature sensitive defect under glucose growth conditions, a phenotype not observed with a *CCR4* deletion. We interpret these results as evidence that CAF1 is part of the CCR4 containing complex but that its function may be somewhat different from that of CCR4.

Previous characterization of mutations in *POP2* (*CAF1*) indicated that CAF1 augmented expression of the glycolytic gene *PGK* specifically during stationary phase,

reduced the derepression of glucose repressed genes like SUC2 and ICL1, and reduced the amount of glycogen and trehalose stored in the cell. In addition to these carbon metabolism and growth defects we observed that CAF1 affected LexA activator induced transcription. The ability of caf1 mutations to suppress spt6 and spt10 defects further indicates the general role in transcription that CAF1 appears to play. These results suggest that CAF1 like CCR4 is a transcription factor required for a diverse set of genes or processes, only some of which are involved in or related to carbon metabolism. While it is unclear as to the specific function of CCR4, CAF1 and their associated factors, the retention of CCR4 on an RNA polymerase II CTD affinity column supports the role of CCR4 as a component of the general transcriptional machinery. Although CCR4 and CAF1 are clearly not core or integrated components of the basal transcription complex whose deletions would render the complex inactive, they do appear to be involved in peripheral and perhaps activated transcription processes. Further characterization of the CCR4 complex and its interaction with transcriptional components will shed light on the function of the apparently evolutionarily conserved CAF1 proteins and CCR4.

## GENERAL DISCUSSION

Transcription by RNA polymerase II is a complex process involving multiple components. Understanding the process involves identifying the pieces of the transcription puzzle as well as understanding the relationships between and the precise function of each of those pieces. This work with the yeast CCR4 protein suggests that CCR4 may be an important part of this puzzle. The fact that it is associated with a protein (yCAF1) which has strong homology to proteins from a variety of other eukaryotic organisms suggests that the complex CCR4 is part of plays a key role in gene regulation. A role that has been conserved through evolution.

Studies of gene regulation have established that transcription in eukaryotic cells is mediated through a multifactor, nucleoprotein group of components. The prevailing hypothesis has been that the complex assembles on promoter DNA from free factors in an ordered stepwise fashion. This view, largely supported by biochemical evidence, has possibly hindered our approach to eukaryotic transcription. This notion of assembly on the template DNA is now coming under challenge from a variety of researchers. Recently, there have been reports of the purification of a largely preassembled RNA polymerase II transcription complex from crude extracts of *Saccharomyces cerevisiae* (Koleske and Young, 1994). This holoenzyme, as it has been dubbed, is the biochemical outcome of a genetic phenotype supporting its relevance *in vivo*. The work started by isolation of suppressor mutations, called SRBs



(suppressor of RNA polymerase B), of the cold sensitive phenotype elicited by partial deletion of the carboxyl-terminal domain of RNA polymerase II (Thompson et al., 1992). Soon afterwards it was shown that these suppressor mutants encoded proteins which copurified in a stable complex with RNA polymerase II (Thompson et al., 1993). Many of the polypeptides in the complex are undefined at this point in time. Some may be the pleiotropic regulatory molecules identified in other yeast genetic screens. The work has the potential to alter the way we think about eukaryotic transcription. It may well be that activators will recognize one component of the holoenzyme and in cooperation with TFIID recruit it to the promoter.

This concept of a holoenzyme also has potential relevance to our work with the CCR4 protein and the CCR4 associated factors. Recently it has been shown that CCR4 is specifically retained by a GST-CTD column, like the SRBs. Indicating that CCR4 may be associated with the holoenzyme. It is unclear why in our immunoprecipitations we are unable to identify these proteins. It may be that the complete integrity of the complex may depend on the method of fractionation or alternatively it is because only a small proportion of the total cellular Pol II is in the complex (Koleske and Young , 1994). In a two hybrid type screen for proteins which are associated with CCR4 we have identified a variety of different classes of factors. Many of these may be factors associated with RNA polymerase II. Further experiments will help to clarify the relationship between CCR4, the CCR4 associated factors, and RNA polymerase II.

CCR4 when bound to LexA can function as an activator of transcription and this activation potential appears to be dependent upon the leucine rich-repeat of CCR4. In light of the result that CCR4 may be bound to Pol II, It may be that what is happening is that CCR4 when bound to DNA via LexA is acting to recruit the RNA polymerase II holoenzyme to a favorable position around the GAL1 promoter of the reporter gene. The LexA-CCR4 fusion appears to be functioning in yeast as it can complement a ccr4-10 allele and other results have shown it to be complexed with the 185 and 195 kDa proteins.

The genetic relationship between the SNFs, SWIs, SPT6, SPT10, and CCR4 genes would lead one to think that CCR4 has a role in chromatin structure. Since CCR4 and now yCAF1 are the only known suppressors of the spt6 and spt10 phenotypes it is tempting to suggest that CCR4 is involved in recognizing proper chromatin structure for transcription initiation. Mutations in CCR4 would therefore act to reduce the formation of these initiation complexes at promoters which are in the active state.

The above theories are somewhat speculative in their nature, but it is clear that CCR4 is part of a multisubunit complex in yeast. This complex has conserved elements within it suggesting an important role for it in eukaryotic transcription. While it is clear that CCR4 when bound to DNA via the LexA moiety can activate transcription, the mechanism responsible for this activation is not. LexA-CCR4 is probably not activating in the same manner as proteins like ADR1 or GAL4 do. The activation domains at the N-terminus of CCR4 are of interest, but once again the

mechanism behind this activation potential is not clear. A better understanding of the proteins associated with CCR4 as well as studies to examine the chromatin structure in strains deficient for CCR4, SPT6 and SPT10 will help to shed light on this question.

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## APPENDIX

**CCR4 is a glucose-regulated transcription factor whose leucine-rich repeat binds several proteins important for placing CCR4 in its proper promoter context**

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**Short Title: Proteins binding leucine-rich repeat of CCR4**

## ABSTRACT

The yeast CCR4 protein is required for the expression of a number of genes involved in non-fermentative growth, including glucose repressible ADH2, and is the only known suppressor of mutations in the SPT6 and SPT10 genes, two genes which are believed to be involved in chromatin maintenance. We show here that although CCR4 did not bind DNA under the conditions tested, it was able to activate transcription when fused to a heterologous DNA binding domain. The transcriptional activation ability of CCR4, in contrast to many other activators, was glucose regulated. Two activation domains were identified, one of which was glucose responsive and encompassed a glutamine-proline rich region similar to that found in other eukaryotic transcriptional factors. The two transactivation regions, when separated from the leucine-rich repeat and the C-terminus of CCR4, were unable to complement a defective ccr4 allele, suggesting that the leucine-rich repeat and the C-terminus make contacts that link the activation regions to the proper gene context. Native immunoprecipitation of CCR4 revealed that CCR4 was complexed with at least four other proteins. The leucine-rich repeat of CCR4 was both necessary and sufficient for interaction with at least two of these factors. We propose that the leucine rich-repeat links CCR4 through its associated factors to its promoter context at ADH2 and other loci where it is required for proper transcriptional regulation.

## INTRODUCTION

The general transcription factor CCR4 from Saccharomyces cerevisiae is required for the transcription of a number of genes involved in non-fermentative growth including that of the ADH2 gene (encoding the glucose-repressible alcohol dehydrogenase II, ADH II) (11). Strains containing deletions of CCR4 also fail to grow at elevated temperatures on non-fermentative medium, consistent with the global role played by CCR4 under these growth conditions (14). Cells lacking CCR4, however, display other phenotypes suggesting that CCR4 is involved in processes in addition to that of controlling non-fermentative growth. ccr4 mutations display a cold sensitivity phenotype under glucose growth conditions, a phenotype observed for defects in transcription initiation complex factors such as TFIIB, RPB1, SRB2, and SRB4 (1,2,29,30,33,34,37,41). In addition, CCR4 is required for the elevated expression at the ADH2 locus and for the altered transcriptional initiation at the his4-912delta locus that results from defects in the SPT6 and SPT10 genes (14). The SPT6 and SPT10 genes encode factors that are responsible for maintaining proper transcriptional control over a wide range of genes and SPT6 has been specifically implicated in the maintenance of chromatin structure (11,26,27,39,40).

spt6 mutations, moreover, suppress defects in the global transactivators SNF2 and SNF5 which are known to be involved in maintaining proper nucleosome positioning (5,6,28,43). Mutations in SNF2 and SNF5 affect the expression of many genes, cause defects in non-fermentative growth and have been shown to be required for the activity of specific DNA-binding transcriptional activators (22). CCR4, the

only known suppressor of spt6, may be functioning to counteract the repressive effects that SPT6 has on chromatin structure. CCR4 is also required for ADR1-dependent ADH2 expression (11). ADR1 is a DNA binding (16) gene-specific activator of ADH2 (15). It is possible that, like the SNF2 and SNF5 proteins, CCR4 functions both in aiding gene specific activators and in overcoming repression by nucleosomes (22,20).

The CCR4 protein contains several regions which may be important to its function. The N-terminal region is rich in glutamines and asparagines which is characteristic of a number of eukaryotic proteins involved in transcription (25). The C-terminus contains a region of homology to the manganese binding site of xylose isomerases (unpublished observations) (10,20). The central region of the protein (residues 350-473) contains five tandem copies of a leucine-rich repeat. Leucine-rich repeat structures have been implicated in protein-protein interactions (3,24,38). Deletions within the CCR4 leucine-rich repeat abolish CCR4 function (25), suggesting that the interaction of the leucine-rich repeat with another protein is important to CCR4 activity. The possibility that CCR4 binds other proteins is consistent with several lines of evidence that suggest that general transcription factors function as components of large multi-subunit complexes, such as observed for the SPT4, -5 and -6 proteins and the SWI1, SWI3, SNF2, and SNF5 proteins (32,40).

We present evidence that CCR4 when bound to DNA via the LexA DNA binding protein activates transcription in a glucose-responsive manner. Two activation domains were identified, neither of which were capable of substituting for

CCR4 function in vivo. CCR4 itself was unable to bind DNA suggesting that it binds other proteins. CCR4 was found to be part of a multi-protein complex and the leucine-rich repeat region was necessary and sufficient for CCR4 interaction with at least two components of this complex. These data suggest that the leucine-rich repeats make protein contacts which bring CCR4 to its proper promoter context.

## Materials and Methods

### Plasmids

LexA202 and LexA87 plasmids (Figure 1A) are 2 um plasmids and have been previously described (4,36). pLexA202-1 is the same plasmid as LexA(1-202)+pL (36). The 1840 and 34 reporter plasmids are 2 um based plasmids with one and eight LexA operators, respectively, controlling the lacZ gene (4,7). LexA-GAL4 contains residues 77-881 of GAL4 (22) and LexA B42 contains an E. coli derived polypeptide that activates transcription in yeast (36).

### Plasmid Constructions

The full-length LexA-CCR4(1-837) fusions were constructed by placing an EcoRI-BglII fragment from plasmid TM7 that contains residues 1-837 of CCR4 (25) into the EcoRI-BamHI polylinker sites of the vectors pLexA202-1 and pLexA87-1. These LexA-CCR4 derivatives are designated pMD18 and pMD7, respectively (See Figure 1). The EcoRI site in each of these plasmids was made blunt with the large subunit



of *E. coli* DNA polymerase (Klenow) to place CCR4 coding sequences in frame with LexA. Deletion of amino acids 14-209 of CCR4 was accomplished by removing an *Apal* fragment from the above described two constructions. Truncation of CCR4 at amino acid 669 was conducted by filling in with Klenow the internal *BamHI* site at bp 2004 of the LexA-CCR4(1-837) fusions, which results in 4 non-CCR4 amino acids RSKI at the C-terminus. Construction of the LexA-leucine-rich repeat fusions were accomplished by digesting plasmid TM5 (pUC18 containing a *HindIII* (cuts at bp 653) to *BamHI* (cuts at bp 2004) fragment of CCR4) with the *TaqI* enzyme and then filling in the resulting 5' overhangs with Klenow. The *TaqI* fragments were subsequently cloned into pUC18, and after sequencing, one clone with an insertion containing base pairs 985 to 1421 of CCR4 was selected for further manipulation. A *BamHI*-*EcoRI*(filled with Klenow) fragment encompassing the leucine-rich repeats (residues 330-474) was then ligated into the *BamHI*-*Sall* (filled with Klenow) sites of both the pLexA87-3 and pLexA202-3 vectors. The *BamHI* site was subsequently cut, filled in with Klenow, and religated to obtain the proper reading frame. The LexA fusion containing amino acids 404-837 of CCR4 was made by removing an *EcoRI*-*BamHI* fragment from pMD18 and substituting *MunI*-*BamHI* fragment from TM5 (see above description of TM5). The LexA-CCR4 fusions expressing amino acids 1-345 and 1-160 of CCR4 contain stop codons at base pair 1033 and 478, respectively. The isolation of these fusions will be described elsewhere. LexA-CCR4-1-13/210-345 was made by removing an *Apal* fragment from the plasmid containing LexA-CCR4-1-345. The LexA-CCR4-1-13 fusion plasmid was constructed by filling in with Klenow the

Bsp120I site at bp 39 of the LexA-CCR4-1-837 plasmids, followed by religating. The LexA-CCR4-1-13 fusion has 58 non-CCR4 amino acids, PRTSGCTTSFAAHRFP-AISHCRSLQITGRLHKASLDGNGRHSRQQDDSKETTTYGR at its C-terminus.

#### Yeast strains, growth conditions, and enzyme assays.

Yeast strains are listed in Table 1. Conditions for growth of cultures on minimal medium lacking uracil and histidine or YEP medium (2% Bactopeptone, 1% yeast extract, 20 mg/liter each of adenine and uracil containing either 8% glucose or 2% ethanol as a carbon source) have been described (7). YD solid medium contained YEP supplemented to 2% glucose and 2.5% agar.  $\beta$ -galactosidase assays were conducted on yeast extracts as described (7). Because yeast expressing LexA-CCR4 fusions proteins were observed to undergo loss of activity with prolonged maintenance on selective plates, assays were conducted within as short as time as possible on new transformants or freshly streaked-out colonies.

#### Native immunoprecipitations

Cells were grown in 2 liters of either YEP or an appropriate selective medium containing 4% glucose or 3% ethanol as described above. Cells were harvested at a density of  $5 \times 10^7$  by centrifugation, were washed in 20 ml of ice cold water, and were resuspended in two volumes of native extract buffer (25 mM  $\text{KPO}_4$  buffer, pH 7.6/150 mM KCl/1 mM NaPPi/1 mM NaF/5 mM  $\text{MgCl}_2$ / 1 mM EDTA/10% Glycerol/1% NP-40/8  $\mu\text{M}$  Leupeptin/3  $\mu\text{M}$  Pepstatin A/1 mM PMSF) prior to lysis in

a bead beater according to manufacturers specifications. The resulting lysates were spun in a microfuge for 15 minutes at maximum speed. The supernatant was then mixed with pre-immune sera coupled to protein A agarose (or Protein A agarose alone) at a 2:1 ratio and incubated at 4°C for 15 minutes with gentle rocking. After clarification in a clinical centrifuge the pre-cleared extract was mixed with anti-sera coupled to protein A agarose at a 5:1 ratio. The immune beads and the supernatant were incubated for 45 minutes at 4°C with rocking. Immune beads were washed three times with 10 volumes of ice cold native extract buffer and twice in 10 volumes of ice cold triton wash buffer (20 mM NaPO<sub>4</sub> buffer, pH 7.6/140 mM NaCl/1 mM EDTA/0.5% Triton X-100). The samples were then subjected to SDS-PAGE and analyzed by Western or silver staining as described (42,44).

#### DNA binding assays

Gel retardation conditions, preparation of extracts, and incubation of extracts with DNA were as described (8). Gel mobility shift assays were conducted with a radio labeled Sau3AI-EcoRV fragment of the ADH2 promoter (bp -329 to +57). The DNA fragment was labeled using DNA polymerase I large fragment (Klenow) with <sup>32</sup>P, which resulted in average activities of 30,000 counts/min/ng. The DNA-binding mix contained the following final concentrations: 2μg of total protein, 100 mM KCl, 10% glycerol, 0.1 mM ZnSO<sub>4</sub>, 0.1 μg/μl poly(dI-dC), 0.1 ng/μl probe in a 10μl final volume. The mix was incubated 20 min on ice before being loaded onto either a 4 or 6% polyacrylamide gel containing 2% glycerol and 2% Ficoll 400 that had been pre-

electrophoresed at 100 V for 60 min at 4 °C in running buffer consisting of 190 mM glycine and 20 mM Tris, pH 8.0. The samples were subjected to electrophoresis at 400 V for 5 min and then 100 V for 3 h before the gel was dried and exposed for autoradiography. Extracts that were used in the incubations were from the following strains: MD9-7c (ccr4), MD9-7c+ (CCR4), and EGY188 containing the MD17 (LexA-CCR4) plasmid. Partially purified CCR4 was prepared from strain EGY188 that expressed a GST-CCR4 fusion protein. Purification of GST-CCR4 will be described elsewhere.

## RESULTS

### **LexA-CCR4 transcriptional activation is carbon source regulated.**

The ability of CCR4 to activate transcription was monitored independent of the ADH2 promoter context using the LexA in vivo transcription assay (4). The complete coding sequence for the yeast CCR4 protein was fused in frame with the coding sequence for the bacterial DNA binding protein LexA (see Figure 1A). We expressed two versions of a LexA-CCR4 fusion protein, one containing only the DNA binding domain of LexA (residues 1-87) and one containing the complete LexA protein (residues 1-202). Amino acid residues 88-202 of LexA contain a dimerization domain which is required for efficient DNA binding (17). The LexA-CCR4 fusion proteins expressed from these plasmids provided CCR4 function as assayed by complementation of the ccr4-1Q allele which causes a temperature sensitive defect under non-fermentative growth conditions (Figure 2). Also, both the 1-87 and 1-202

LexA-CCR4 fusion constructs complemented the inability of the strain carrying the ccr4-10 allele to grow at 16°C on medium containing glucose (data not shown).

The LexA-CCR4 constructs were transformed into yeast along with a GAL1-lacZ reporter gene under the control of the LexA operator (Figure 1B). Both versions of the LexA-CCR4 fusion proteins activated expression of a GAL1-lacZ reporter gene containing either single or multiple LexA operators upstream of the GAL1 promoter (Table 2). However, LexA-CCR4 activated transcription to a much greater extent under non-fermentative growth conditions than under glucose repressed conditions (Table 2). No activation by CCR4 was detected from the GAL1-lacZ target gene LR1Δ1 (Figure 1) that lacked a LexA operator (data not shown) nor could the LexA DNA-binding domain alone activate expression from the reporters (Table 2). LexA-CCR4 protein concentration was found to be two-to three-fold lower under non-fermentative conditions than under glucose repressed conditions (Figure 3, compare lane b to lane a), suggesting that the ability of CCR4 to activate transcription in a carbon source regulated manner was even greater than that observed in Table 2. While it is possible that the increased abundance of LexA-CCR4 under glucose growth conditions was titrating a factor required for its transcriptional activity, this seems unlikely. LexA-CCR4 activity would have been expected to increase dramatically in a strain lacking the genomic CCR4 gene. This was not observed, however, in which LexA-CCR4 activity in a CCR4 background was equal to or greater than that observed when LexA-CCR4 was placed in a ccr4 strain background (data not shown). Western analysis also indicated that the LexA-CCR4 fusion

proteins were present at only slightly elevated levels under glucose growth conditions as compared to wild-type CCR4 levels and at lower levels than wild-type CCR4 under non-fermentative growth conditions (Figure 3), suggesting that the ability of LexA-CCR4 to activate transcription was not due to an artificial overproduction of CCR4 protein. These results suggest that CCR4 when bound to DNA can function to activate transcription in a carbon-source regulated manner.

In contrast to CCR4 other known transcriptional activators fused to LexA did not display carbon source regulation. Several LexA-ADR1 derivatives, LexA-GAL4, and LexA-B42 (an *E.coli*-derived activator) all showed a reduction in transcriptional activity under non-fermentative growth conditions (Table 2) (7, 23,36). It has also been previously reported that the activation potential of the general transcriptional factors SNF2 and SNF5, which like CCR4 are required for non-fermentative gene expression, were not glucose regulated when fused to LexA (23). The decrease in the ability of these other LexA-fusion proteins reported in Table 2 to activate under non-fermentative growth conditions is likely due to their being under the control of the ADH1 promoter (Figure 1), which is derepressed on glucose and repressed under non-fermentative conditions (13). Western analysis confirmed a three- to five-fold reduction in the LexA-ADR1 proteins, a five-fold reduction in the LexA-B42 activator, and a three-fold reduction in the amount of LexA-GAL4 present under non-fermentative growth conditions as compared to glucose growth conditions (7; data not shown).

Many transcriptional factors contain sequence specific DNA binding domains. We

therefore examined whether CCR4 could bind DNA by using a gel-retardation assay used to identify ADR1 binding to ADH2 sequences (8). We did not detect binding of CCR4 to ADH2 regulatory sequences (bp -329 to +57) when using extracts from strains overexpressing CCR4 or extracts containing partially purified CCR4 (data not shown). Further, we saw no differences in the association of factors with ADH2 regulatory sequences when extracts from CCR4<sup>+</sup> or ccr4<sup>-</sup> containing strains were compared (data not shown). Although the proper conditions for CCR4 binding to DNA may not have been uncovered, these results suggest that CCR4 neither binds DNA nor affects the binding of other factors to the ADH2 promoter.

#### **CCR4 contains two activation regions.**

The importance of the amino terminal region of CCR4, which is rich in glutamines and prolines, to CCR4 function was examined by deleting amino acids 14-209. LexA(1-87)-CCR4-1-13/210-837 was able to activate transcription of the GAL1-lacZ reporter gene under both fermentative and non-fermentative growth conditions although its ability to activate under non-fermentative growth conditions was greatly diminished relative to full-length LexA-CCR4 (Figure 2). This result implicates the 14-209 region as being responsible for the increased activity of LexA-CCR4 under non-fermentative conditions. It also suggests, however, that this is not the sole activation region present in CCR4. When the glutamine rich region alone (residues 1-160) was fused to LexA, it functioned as an activator in a glucose-responsive manner, indicating that it did indeed contain an activation domain that was carbon-source

dependent. The glucose-responsiveness of LexA-CCR4-1-160 was not apparent when a reporter containing eight LexA operator sites was used (Figure 2). Deletion of residues 14-209, had no apparent effect, however, on the ability of LexA-CCR4 to complement a ccr4-10 allele, again suggesting the presence of a second activation region in CCR4 (Figure 2). A second transcriptional activation domain that was not glucose repressed was identified between residues 210 and 345 as demonstrated with LexA(1-202)-CCR4-1-13/210-345 (Figure 2). Putting the two domains together (see LexA(1-202)-CCR4-1-345) resulted in a synergistic increase in transcriptional activation.

In contrast, the leucine-rich repeat and the C-terminus of CCR4 were incapable of activating transcription when fused to LexA (Figure 2). In order to determine if the two LexA derivatives which lacked activation potential were still capable of binding the LexA operator, a transcription interference assay was used to monitor DNA binding of the fusion proteins. The interference assay utilizes the pJK101 plasmid which carries 2 LexA operators placed between the GAL4 UAS binding site and the LacZ reporter (Figure 1B) (18). Under galactose growth conditions GAL4 binds to its UAS site and activates transcription of the reporter. Binding of LexA-fusion proteins to the LexA operator reduces this GAL4-induced transactivation. LexA fusions which were incapable of activation were examined using this assay. Both the LexA(1-202)-CCR4 derivatives containing only the leucine rich-repeats or the C-terminus of CCR4 were capable of interfering with the GAL4 induced expression by at least three fold (Figure 4). These data indicate that their



inability to activate is not due to defects in binding to the LexA operator.

Each of the two activation domains was found to be much more active than the full-length LexA-CCR4 fusion (See Discussion). LexA-CCR4-1-345, -1-160 or -1-13/210-345 encompassing both or each activation domain failed, however, to complement the ccr4-10 allele (Figure 2). These results suggest that while residues 1-345 of the CCR4 protein are sufficient for transcriptional activation it is likely that the leucine-rich repeats and the C-terminus are required for placing these activation regions in the proper promoter context for CCR4 function.

#### **CCR4 is required for ADR1 activation of transcription.**

The observation that CCR4 displays a carbon-source regulated transcriptional activation ability when bound to DNA suggests that it may function as a co-activator with ADR1. We therefore examined the effect of a ccr4 mutation on LexA-ADR1 activation of transcription. The ccr4-10 allele had a 2.4 fold effect on the ability of LexA-ADR1-1-1323 (full length) protein fusion to activate. Since ADR1 contains three and possibly four functionally redundant activation regions, designated TADI through TADIV (7), we assessed whether LexA-ADR1 fusions containing fewer domains were also sensitive to the ccr4-10 allele. As shown in Table 3 the ccr4-10 allele also reduced by two-fold the ability of LexA-ADR1-1-642 (contains three regions important to ADR1 activation function) to activate. LexA-ADR1 fusions which contained only one activation domain were more sensitive to a ccr4-10 mutation. The activation ability of LexA-ADR1-359-740 (contains only TADIII),

LexA-ADR1-148-359 (contains only TADII), and LexA-ADR1-1-220 (contains only TADI) were reduced five to eleven fold in the ccr4-10 background. In comparison, the ccr4-10 allele affected LexA-GAL4-74-881 (22) mediated activation by 1.5 fold and had no effect on the E.coli-derived activator LexA-B42 (36). These results support the hypothesis that CCR4 may play a role in assisting ADR1 activation but also clearly indicate that CCR4 does not play an essential role.

#### **ADR1 is not co-immunoprecipitated with CCR4**

To determine if the requirement of CCR4 for ADR1 activation reflected a physical interaction between CCR4 and ADR1, we examined by immunoprecipitation the direct association of these two factors. ADR1 was immunoprecipitated from a native extract using antibody raised against an ADR1 C-terminal peptide. The precipitated proteins were subjected to Western analysis using an antibody raised against a CCR4 N-terminal synthetic peptide. ADR1 was specifically immunoprecipitated as detected with anti-ADR1 antibody (Figure 5, lane a) and failed to be precipitated when either preimmune sera or immune sera pre-treated with excess antigen was used (42). CCR4 antibody, however, failed to detect CCR4 in the immunoprecipitates (Figure 5, lane a) although CCR4 was present in the crude extract (Figure 5, lane b). We repeated these experiments using CCR4 antibody to conduct the initial immunoprecipitation and anti-ADR1 antibody for detecting the presence of ADR1 following Western analysis. In this case, a strain carrying multiple copies of the ADR1 gene was used to maximize the likelihood of detecting ADR1 in the

immunoprecipitates. CCR4 was specifically precipitated by the anti-CCR4 peptide antibody (Figure 5, lane c) but failed to be precipitated when preimmune sera or immune sera pre-incubated with excess peptide was used (25). ADR1, however, was not detected among the CCR4 immunoprecipitated proteins (Figure 5, lane c) although it was present in the crude extract. These results indicate that CCR4 and ADR1 are not tightly associated *in vivo*.

### **Immunoprecipitation with CCR4 antibody reveals a multi-protein complex**

Since the leucine-rich repeat is a putative protein binding domain and is required for CCR4 transcriptional function, we examined the possibility of a direct physical interaction between CCR4 and proteins other than ADR1 by determining which proteins co-immunoprecipitated with CCR4. Native extracts were treated with anti-CCR4 antibody and the resultant immunoprecipitated proteins were subjected to SDS-PAGE and silver staining. Two abundant species were co-immunoprecipitated with CCR4 (Figure 6A, lane b). These proteins had molecular weights of 195 kDa and 185 kDa, respectively. Each was absent when preimmune sera or immune sera blocked with excess peptide was used (Figure 6A, lane a and c, respectively). The presence of CCR4 in the immunoprecipitate was confirmed by Western analysis using the same samples (data not shown) and could also be visualized by silver staining (Figure 6A, lane b). The 195 and 185 kDa species were not immunoprecipitated when native extracts of a strain lacking CCR4 (ccr4-10 allele) were treated with anti-CCR4 antibody (Figure 6A, lane e). The protein band in lane e in the 180-190 kDa

region did not comigrate with the 185 kDa protein (see also Figure 6B). These results indicated that CCR4 is associated with at least two other proteins to form a protein complex in vivo. Two additional proteins of molecular weights 116 and 140 kDa were also observed to co-immunoprecipitate with CCR4 (Figure 6B lane b). The association of the 116 and 140 kDa polypeptides with CCR4 varied depending on the experiment, suggesting that they were less tightly associated with CCR4 than the 185 and 195 kDa proteins.

#### **The leucine-rich repeats are necessary and sufficient for formation of the CCR4 protein complex**

To identify which regions of CCR4 were required for binding the 195 and 185 kDa proteins, native extracts from strains containing alleles deleted for different regions of CCR4 were treated with anti-CCR4 antibody. The immunoprecipitated proteins were treated as described above and the 195 and 185 kDa species were detected by silver staining. As shown in Figure 7, lanes b and d, the 195 and 185 kDa proteins were co-immunoprecipitated with CCR4 when residues 14-209 or 670-837 were removed from CCR4, respectively. The ccr4-1-669 allele results in phenotypes indistinguishable from that of a ccr4 deletion and a LexA-CCR4-1-669 fusion was transcriptionally inactive (data not shown). When portions of the leucine-rich repeat region were deleted, however, the 195 and 185 kDa proteins were not co-immunoprecipitated (Figure 7, lane f and g). The CCR4- $\Delta$ 219-394 and CCR4- $\Delta$ 393-

456 proteins were present in the immunoprecipitates in an abundance comparable to that observed for the undeleted CCR4 proteins (25). These results indicate that the leucine rich repeats are essential for the association of CCR4 with the 195 and 185 kDa proteins *in vivo*.

We subsequently examined the ability of the leucine rich repeats by themselves to form a complex with the 195 and 185 kDa proteins. A portion of the CCR4 gene containing the coding sequence for the leucine-rich repeats (residues 350-465) as well as a small amount of flanking sequences (total residues 330-474) were fused to the LexA(1-87) protein (designated LexA-LRR). The plasmid expressing the fusion protein was transformed into a yeast strain carrying a ccr4-10 allele and into a strain carrying a wild-type CCR4 gene. Immunoprecipitation of the fusion protein from native extracts of transformants expressing the LexA-LRR protein was carried out using an antibody raised against the LexA protein. The resultant proteins were subjected to SDS-PAGE and were visualized by silver staining. The 195 and 185 kDa proteins were co-immunoprecipitated in the presence of the LexA-LRR fusion protein (Figure 8, lane a) but not from extracts that lacked the LexA-LRR (data not shown). This result demonstrates that the leucine-rich repeats are responsible for the interaction between CCR4 and the 195 and 185 kDa proteins. When wild type CCR4 and LexA-LRR protein co-existed in the cell, the amount of 195 and 185 kDa proteins that co-immunoprecipitated with the LexA-LRR fusion protein was much less than that immunoprecipitated with LexA-LRR in a strain lacking CCR4 (Figure 8, compare lane b to lane a). This indicates that LexA-LRR binds the 185 and 195 kDa proteins

less well than full-length protein (also compare lane c to lane a).

Because CCR4 transcriptional activity is regulated by glucose (Table 2) and CCR4 is specifically required for non-fermentative gene expression, we wished to determine if the proteins associated with CCR4 did so in a carbon source dependent manner. Native extracts from a wild-type strain of CCR4 following growth on glucose and ethanol were immunoprecipitated with anti-CCR4 antibody. Both the 195 and 185 kDa proteins were found to be co-immunoprecipitated with CCR4 using extracts taken under both growth conditions (Figure 9, lane a and b respectively). The 140 and 116 kDa species that were shown to be co-immunoprecipitated with CCR4 (Figure 6B) were also complexed with CCR4 irrespective of carbon source (data not shown). The decreased and varied abundance of the 140 and 116 kDa proteins in the immunoprecipitates precluded the determination of the specific region of CCR4 with which they interacted.

## DISCUSSION

### **CCR4 displays a glucose regulated transcriptional activation ability**

Our results indicate that the yeast CCR4 protein is capable of activating transcription in a glucose-repressible manner when brought to the DNA through a heterologous DNA-binding protein. CCR4 itself, however, was not found to bind DNA. The ability of the LexA-CCR4 fusion proteins to function as an activator correlated with

their ability to allow non-fermentative growth at elevated temperatures and to allow growth on glucose containing medium at 16°C (Figure 2; 25). For example, both leucine-rich repeat deletions and the C-terminal deletion of residues 670-837 inhibited CCR4 transcriptional activity as well as its ability to complement a ccr4-10 mutation. These results indicate that LexA-CCR4 function in the LexA transcription assay mimics CCR4 function *in vivo*. The implication, therefore, is that there exists a mechanism by which CCR4 is brought to the vicinity of the DNA. This function is perhaps served by the factors which are complexed with CCR4. Whether CCR4 is a component of a transcriptional activator complex as observed for Hap 2,3,4 in which there is a division of DNA binding and activation functions is unclear (18, 31). It is equally possible that CCR4 plays a role modulating chromatin structure or the general transcriptional machinery.

The domain responsible for the glucose regulation of LexA-CCR4 was localized to the N-terminal region of CCR4. Deletion of residues 14 to 209 eliminated the derepression of CCR4 transcriptional activity upon glucose removal (Figure 2) and residues 1-160 (designated TADI) by themselves displayed glucose repressed activation (Figure 2). This N-terminal region like many other eukaryotic transcription factors is rich in glutamines and prolines. While it has been shown that the glutamine-rich activation domain of mammalian protein Sp1 does not function in yeast, it is possible that the CCR4 glutamine-rich region is of a different type than that of Sp1 (35). Our data can not distinguish between the CCR4 region being itself glucose regulatable or binding a protein whose activity is carbon source controlled.

A second transactivation domain was localized to residues 210-345 (designated TADII), that acted synergistically with TADI (Figure 2). The transactivation displayed by TADI and TADII is in some ways different from that displayed by the full-length CCR4. First, the TADI and TADII domains are much more potent activators than full-length CCR4. It is possible that the TADI and TADII domains are more active by themselves because they have been released from an inhibition present in the full-length protein. Alternatively, the TADI and TADII domains may be displaying such potent activation due to their being released from the CCR4 protein complex. Full-length CCR4 transcriptional activation ability may be decreased because of its association with a number of factors that reduce its ability to activate. Second, the TADI and TADII domains show a multiplicative increase in activation potential as the number of LexA operators upstream of the LacZ reporter is increased. This is in contrast to what is observed with full-length CCR4 whose ability to activate increased only slightly with an increase in LexA operator sites. The C-terminal half of CCR4 may interact with a limiting factor causing full-length CCR4 activity to increase only slightly with an increase in LexA operator sites. We believe that the transactivation ability of full-length CCR4 more accurately represents its true function rather than the high values observed for TADI and TADII alone.

It should also be noted that LexA(1-202)-CCR4 was less active than LexA(1-87)-CCR4 in activating transcription (Table 2; Figure 2). One explanation for this difference is that the dimerization of LexA(1-202) interferes with CCR4 activity, perhaps by forcing an unusual conformation upon CCR4 or an unnatural arrangement



of proteins associated with CCR4. This effect appears to be specific to CCR4 since a number of other proteins fused to LexA(1-202) do not display this diminution in activity (7,36; unpublished observations). This decreased activity of LexA(1-202)-CCR4 may account for the relatively lower activity observed for full-length CCR4 fused to LexA as compared to that obtained with the LexA-CCR4 fusions containing just the N-terminal sequence of CCR4.

**The leucine-rich repeat binds two proteins that may be required for presenting CCR4 to its proper promoter context.**

The leucine-rich repeats and the C-terminus of CCR4 are required for CCR4 function but neither region alone could promote transcription. The N-terminal domains described above function in that role, although these regions alone could not complement a ccr4 allele *in vivo*. While it is possible that deletion of the leucine-rich repeats or C-terminus inactivated CCR4 by placing the protein into an improperly folded form, we prefer the alternative explanation that the role of the leucine-rich repeats and the C-terminus of CCR4 is to present CCR4 to its proper place at the promoter and to possibly regulate the N-terminal activation domains. The identification of two proteins, 195 and 185 kDa in size, that form a stable complex with the leucine-rich repeats of CCR4 reinforces this notion. The binding of the 195 and 185 kDa proteins to the leucine-rich repeat may make the leucine-rich repeat essential for CCR4 function. Two other proteins, 140 and 116 kDa in size, were also found to associate with CCR4. The site of binding for these latter two was not

determined due to their weaker association with CCR4.

The contact that these 195 and 185 kDa proteins and CCR4 make at the ADH2 promoter remains unclear. CCR4 was shown not to complex with ADR1, although CCR4 was required for maximal expression of LexA-ADR1 fusion proteins. We interpret these data to indicate that CCR4 is not a direct intermediate in ADR1 function, but rather modulates some feature of ADH2 transcription which impinges on ADR1 activation. Since CCR4 is the only known suppressor of the spt6 mutation, a gene presumed to be involved in regulating or maintaining chromatin structure, CCR4 may be required to antagonize the negative effects of SPT6 (and possibly SPT10) on chromatin. The 195 and 185 kDa proteins appear to be neither SPT6 nor SPT10 (12; unpublished observations). It is also possible that CCR4 acts independently of chromatin to promote transcription, perhaps by aiding the action of factors in the initiation complex.

Although a number of leucine-rich repeat proteins have been identified (discussed in ref. 25) in only two cases have the proteins that bind to these regions been identified. The leucine-rich repeat of ribonuclease inhibitor binds ribonuclease (24) and the leucine-rich region of thyrotropin receptor (and its related family members) binds thyrotropin hormone (and the corresponding hormones) (3). Preliminary analysis of contacts between these leucine-rich repeats and their cognate binding proteins have yet to yield a general consensus or structure characteristic of these interactions. The crystal structure of ribonuclease inhibitor indicates that leucine-rich repeats consist of a parallel  $\beta$ -sheet with  $\beta$   $\alpha$  loops, but the precise site of

ribonuclease binding is not known (20). Because CCR4 contains the fewest number of leucine-rich repeats among this family of proteins, identifying its interaction regions with the 185 and 195 kDa proteins and cloning the genes for these proteins should aid in analyzing the protein-protein interactions of this important structural motif.

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Table 1. Yeast Strains

Strain	Genotype
237-1b	<u>MATa</u> <u>adh1-11</u> <u>his3</u> <u>leu2</u> <u>trp1</u> <u>ura3</u>
EGY188	<u>MATa</u> <u>his3</u> <u>leu2</u> <u>trp1</u> <u>ura3</u> <u>Lex<sub>or</sub>-LEU2</u>
MD9-7c	<u>MAT<math>\alpha</math></u> <u>adh1-11</u> <u>his3</u> <u>trp1</u> <u>ura3</u> <u>ccr4-10</u>
MD9-7c+	same as MD9-7c except <u>ccr4-10::CCR4-TRP1</u>
876-2c	<u>MAT<math>\alpha</math></u> <u>adh1-11</u> <u>his3</u> <u>trp1</u> <u>ura1/ura3</u> <u>ccr4-391::HIS3</u> <u>TRP1::ccr4-392/457</u>
906-6	<u>MATa</u> <u>adh1-11</u> <u>his3</u> <u>trp1</u> <u>ccr4-391::HIS3</u> <u>TRP1::ccr4-218/394</u>
86-6d	<u>MATa</u> <u>adh1-11</u> <u>his3</u> <u>trp1</u> <u>ccr4-391::HIS3</u> <u>TRP1::ccr4-1-13/210-837</u>
408-6d-TL5	<u>MAT<math>\alpha</math></u> <u>adh1-11</u> <u>leu2</u> <u>spt10-1</u> <u>ccr4-1-669</u>
411-40	<u>MATa</u> <u>adh1-11</u> <u>adh3</u> <u>his4</u> <u>trp1</u> <u>ura1</u> <u>adr1-1::1-ADR1-TRP1</u>
411-1	same as 411-40 except <u>adr1-1::96-ADR1-TRP1</u>



Table 2. The Transcriptional Activity of LexA-CCR4 Is Glucose Repressible

Fusion Protein	Reporter	$\beta$ -Galactosidase	
	Plasmid	Activity (U/mg)	
	# LexA op	Glucose	Ethanol
LexA(1-87)-CCR4(1-837)	8	9	76
	1	4.8	33
LexA(1-202)-CCR4(1-837)	8	3.2	45
	1	2.9	15
LexA(1-87)	8	< 1.5	< 1.5
	1	< 1.5	< 1.5
LexA(1-202)	8	< 1.5	2.9
	1	< 1.5	< 1.5
LexA(1-202)-ADR1(1-642) <sup>a</sup>	1	1800	460
LexA(1-87)-ADR1 (148-359) <sup>a</sup>	1	33	5.4
LexA(1-87)-B42	8	1100	300
LexA(1-87)-GAL4	1	1000	540

LexA and all LexA fusions were expressed from a 2 $\mu$  plasmid in strain 237-1b which contained either the 1840 reporter which has a single LexA operator controlling lacZ expression or the 34 reporter which contains 8 Lex operator sites (See Figure 1B). Yeast were grown in minimal medium lacking uracil and histidine and which was supplemented with either 8% glucose (Glucose) or 2% glycerol and 2% ethanol (Ethanol). Beta-galactosidase assays were conducted as described in Material and Methods. All values represent the average of at least three separate determination and all SEMs were less than 20%. Western analysis indicated that all LexA-CCR4 fusions were of the expected size and of comparable abundance (data not shown).

<sup>a</sup>Values taken from reference 7.

Table 3. Mutations in CCR4 affect ADR1 activation ability

LexA-Fusion Protein	CCR4 Genotype	$\beta$ -Galactosidase Activity (U/mg)	Fold Decrease in Activation
LexA(1-202)-ADR1(1-1323)	<u>CCR4</u>	1300	2.4
	<u>ccr4-10</u>	540	
LexA(1-202)-ADR1(1-642)	<u>CCR4</u>	1900	2.7
	<u>ccr4-10</u>	700	
LexA(1-202)-ADR1(359-740)	<u>CCR4</u>	780	11
	<u>ccr4-10</u>	74	
LexA(1-202)-ADR1(148-359)	<u>CCR4</u>	1200	4.8
	<u>ccr4-10</u>	250	
LexA(1-202)-ADR1(1-220)	<u>CCR4</u>	2.8	5.6
	<u>ccr4-10</u>	< 0.5	
LexA(1-87)-GAL4	<u>CCR4</u>	1000	1.6
	<u>ccr4-10</u>	640	
LexA(1-87)-B42	<u>CCR4</u>	1200	0.75
	<u>ccr4-10</u>	1600	

$\beta$ -galactosidase activities were conducted as described in Table 2. The 1840 reporter was used in all cases except for LexA (1-202)-ADR1(148-359) and LexA(1-87)-B42 in which the 34 reporter containing 8 LexA operator sites was used. All SEMs were less than 20%. Strain MD9-7c+ (CCR4) and MD9-7c (ccr4-10) are isogenic except for the CCR4 allele.

Figure 1. Plasmids used in the study of transcriptional activation by LexA-CCR4 fusion proteins. A. LexA expression plasmids used to produce LexA-CCR4 fusion proteins. Expression plasmids differed only in polylinker sequences (shown below partial plasmid diagram) into which CCR4 fragments were inserted. pLexA(1-87) is the same as pLexA202-1 except it contains only the N-terminal 87 residues of LexA. The proper reading frame is indicated by codon groups. Restriction enzyme sites that were used in the construction of LexA-CCR4 fusion plasmids are indicated. B. LexA operator-controlled reporter plasmids. Two reporters were used to measure transcriptional activation by LexA-CCR4. Shown above is the 34 reporter containing eight LexA operators. The 1840 reporter (not shown) is identical to the 34 reporter except that it contains a single LexA operator; the second and third plasmids were used to measure LexA-CCR4 binding to the LexA operator; the fourth plasmid was used as a control.

Figure 2. Transcriptional activation by LexA-CCR4 fusion proteins. The solid bar represents CCR4 protein residues fused to either LexA(1-87) or LexA(1-202) as indicated. Strain 237-1b was grown in minimal medium lacking uracil and histidine and supplemented with either 8% glucose (Glucose) or 2% ethanol and 2% glycerol (Ethanol). All strains carried the 34 reporter plasmid which has 8 LexA binding sites with the exception of the values for LexA-CCR4-1-345, LexA-CCR4-1-160 and LexA-CCR4-1-13/210-345 whose upper value was obtained from strains carrying the 1840 reporter (one LexA binding site). We were unable to obtain values for the 34 reporter with the LexA(1-202)-CCR4 (1-345) construct due to poor growth, possibly due to titration of a limiting transcription factor.  $\beta$ -galactosidase units are in U/mg and represent the average of at least three separate determinations. All SEMs were less than 20% except for values less than 20 U/mg in which case SEMs were less than 30%. None of the LexA-CCR4 fusions were able to act with the LexA reporter LR1 $\Delta$ 1 which lacks LexA operator binding sites (Figure 1). Western analysis indicated that all LexA-CCR4 fusions were of the expected size and of comparable abundance (data not shown). Complementation analysis was conducted in strain MD9-7c (*ccr4-10*) which displays temperature sensitive growth at 37°C on non-fermentative medium. Similar results were obtained when the ability to complement the cold-sensitivity phenotype of *ccr4-10* (growth at 16°C on glucose-containing medium) was scored.

\* The 1840 reporter was used.

Figure 3. Carbon source dependent expression of LexA-CCR4. Strain 237-1b containing LexA(1-87)-CCR4-1-837 was grown in glucose or ethanol/glycerol containing medium as described in Figure 2. Western analysis was conducted using crude anti-serum raised against an N-terminal CCR4 peptide (25). G-glucose grown cells; Et-ethanol/glycerol grown cells. CCR4 and LexA-CCR4 proteins are indicated.

Figure 4. Transcription interference assays with LexA-CCR4 derivatives. Yeast strain 237-1b or EGY188 containing the different LexA-CCR4 fusions and the JK101 reporter plasmid were grown in minimal medium lacking uracil and histidine and supplemented with 2% galactose and 3% glycerol. No substantive differences in values were observed between the two strains. SEM values are < 20% except for those assay values under 100 U/mg whose SEMS values were < 25%. None of the LexA fusion proteins affected  $\beta$ -galactosidase activity from plasmid  $\Delta$ 20B that is the same as JK101 except that it lacks the LexA binding site (Figure 1).

Figure 5. ADR1 does not co-immunoprecipitate with CCR4. Western analysis was conducted with both purified ADR1 and CCR4 antibodies for all lanes. The band above the CCR4 protein is a non-specific protein which the anti-CCR4 antibody recognizes in Western analysis. This protein is not immunoprecipitated with this antibody as shown in lane c. Lane a- ADR1 purified antibody to conduct the immunoprecipitation from strain 411-40; lane b- yeast crude extract from strain 411-40; lane c- CCR4 purified antibody to conduct the immunoprecipitation from strain 411-1; lane d- yeast crude extract from strain 411-1.

Figure 6. CCR4 co-immunoprecipitates with several proteins. A. Native immunoprecipitations were conducted as described in Materials and Methods under glucose growth conditions using strain MD9-7c+ (CCR4) or MD9-7c (ccr4-10). Molecular weight markers are indicated on the right. Lanes a and d (P)- incubation with preimmune serum; lanes b and e (E)- incubation with purified CCR4 antibody; lanes c and f (B)- incubation with an excess of N-terminal CCR4 peptide prior to addition of CCR4 antibody. B. Immunoprecipitations were conducted as in panel A. The 195, 185, 140, 116, and CCR4 proteins are indicated in lane b with black squares. Lane a- excess N-terminal CCR4 peptide was added prior to incubation of native extracts from strain MD9-7c+ (CCR4) with purified CCR4 antibody; lane b- same as lane a except no peptide was added; lane c- same as lane b except strain MD9-7c (ccr4-10).

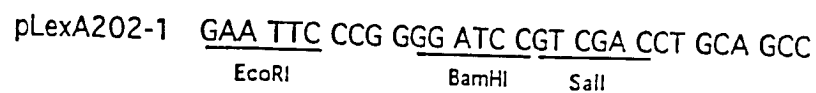
Figure 7. The leucine-rich repeat is required for binding the 185 and 195 kDa proteins. Silver staining and immunoprecipitation of CCR4-associated proteins was conducted as described in Figure 6. E- incubation with CCR4 antibody; B- incubation with excess peptide antigen prior to addition of CCR4 antibody. Lanes a and b- strain 86-6d (CCR4 1-13/210-837); lanes c and d- strain 408-6d-TL5 (CCR4-1-669); lane e- strain MD9-7c+ (CCR4); lane f- strain 906-6 (CCR4-1-217/394-837); lane g- strain 876-2c (CCR4-1-391/455-837). Other experiments indicated that the presence of the *spt10* allele in strain 408-6d-TL5 did not affect CCR4 protein expression (unpublished observations).

Figure 8. The leucine-rich repeat binds the 185 and 195 kDa proteins. LexA-LRR expressed in strain MD9-7c or 237-1b was immunoprecipitated from non-denatured extracts with purified antibody directed against LexA or CCR4 N-terminal peptide as described in Figure 6. Proteins were detected following SDS-PAGE by silver staining. As detected by Western analysis LexA-LRR protein concentration was about two-fold greater than that of CCR4 protein. Lane a-strain MD9-7c (ccr4-10) transformed with LexA-LRR and extracts treated with LexA antibody; lane b-strain 237-1b (CCR4) transformed with LexA-LRR and extracts treated with LexA antibody; lane c- strain 237-1b without LexA-LRR and extracts treated with CCR4 antibody.

Figure 9. Carbon source regulation of proteins binding to CCR4. The figure represents silver staining of proteins co-immunoprecipitating with CCR4 using antibody directed against the N-terminal CCR4 peptide. Cells from yeast strain MD9-7c+ (CCR4) were grown on YEP medium with the appropriate carbon source. G-glucose grown cells; Et-ethanol grown cells; B-excess N-terminal CCR4 peptide was added prior to addition of CCR4 antibody. Lanes b and c-glucose grown cells; lanes a and d-ethanol grown cells.

Figure 10. CCR4 functional domains. CCR4 functional regions are indicated and described in the text.

A.



B.



Figure 2.

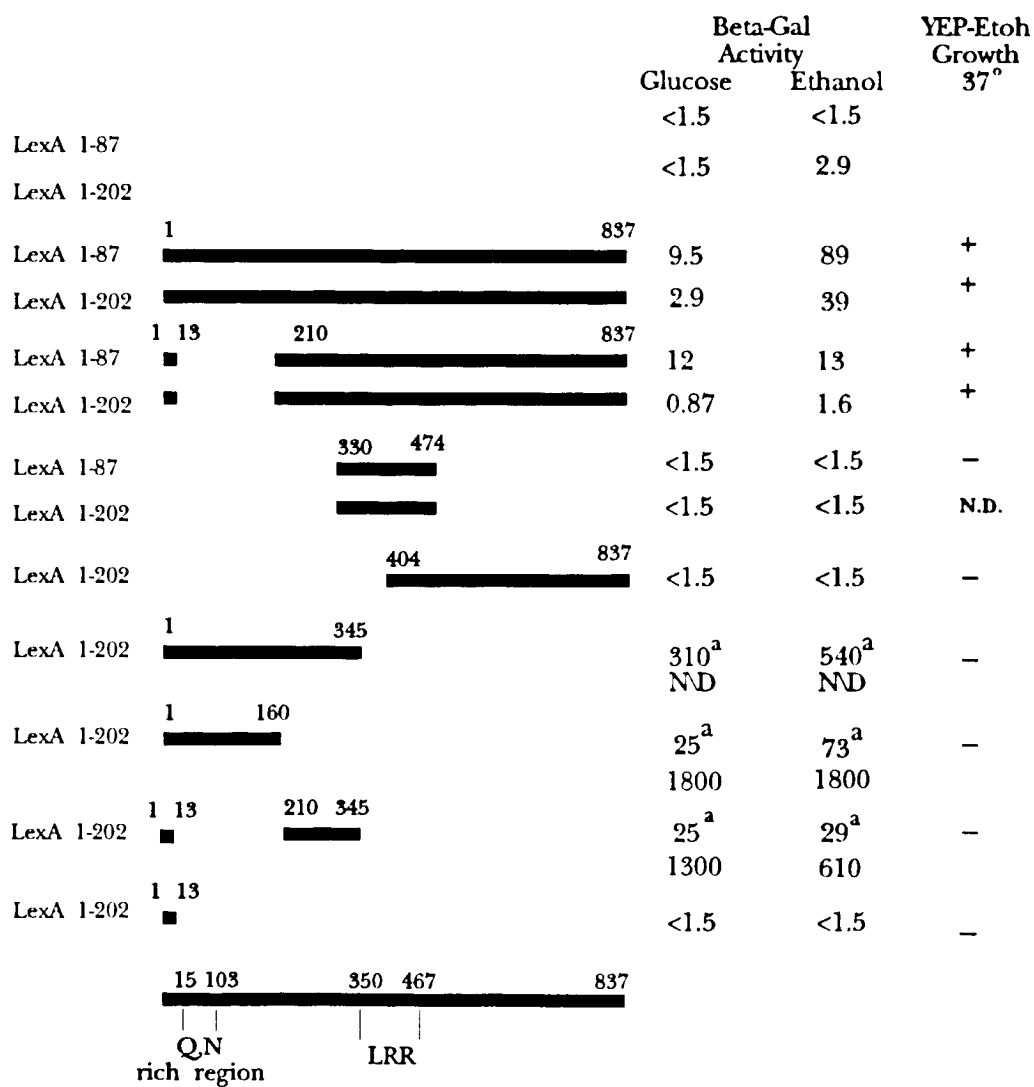


Figure 3.

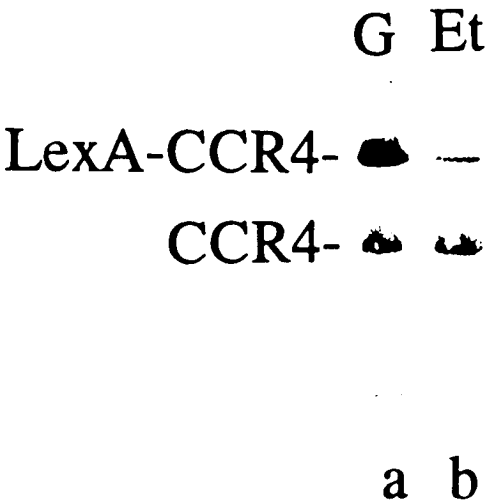




Figure 4.

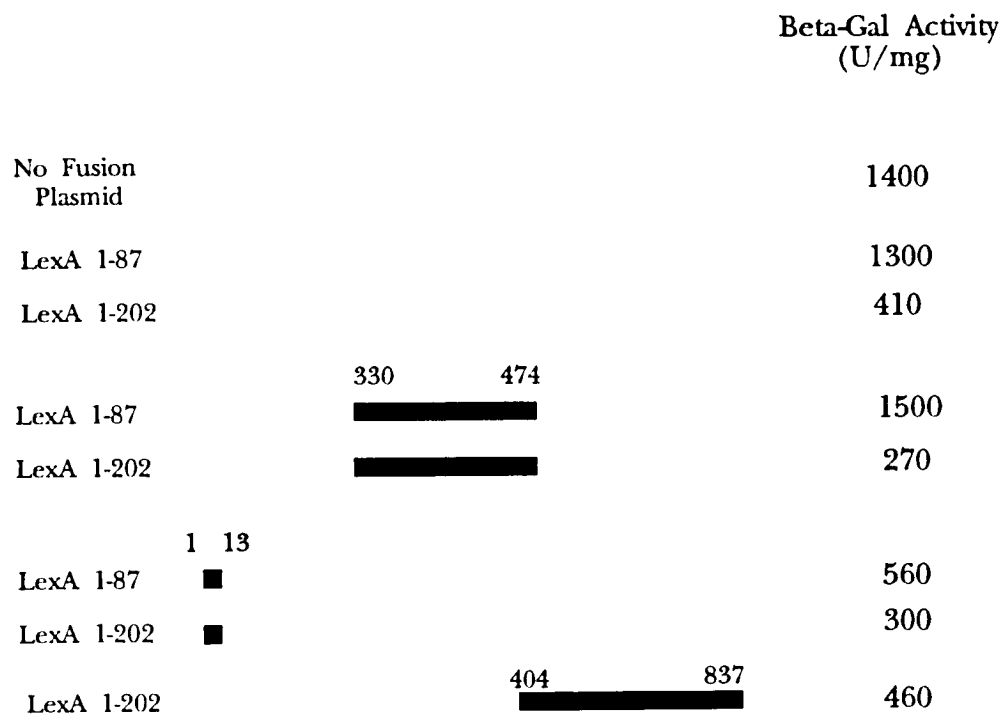


Figure 5.

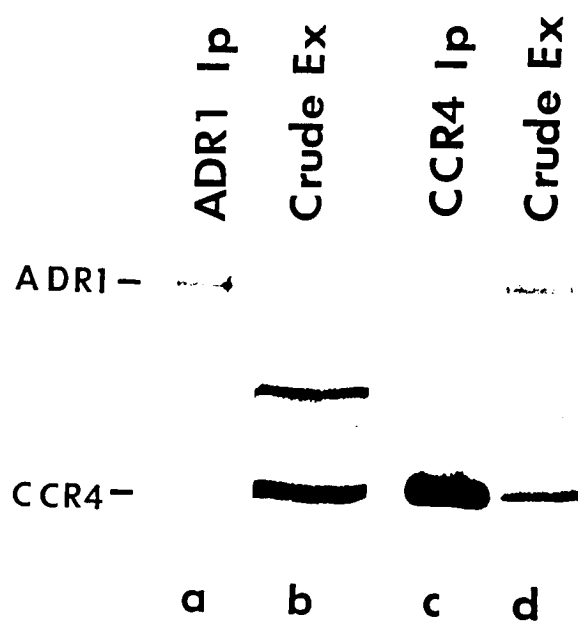


Figure 6A.

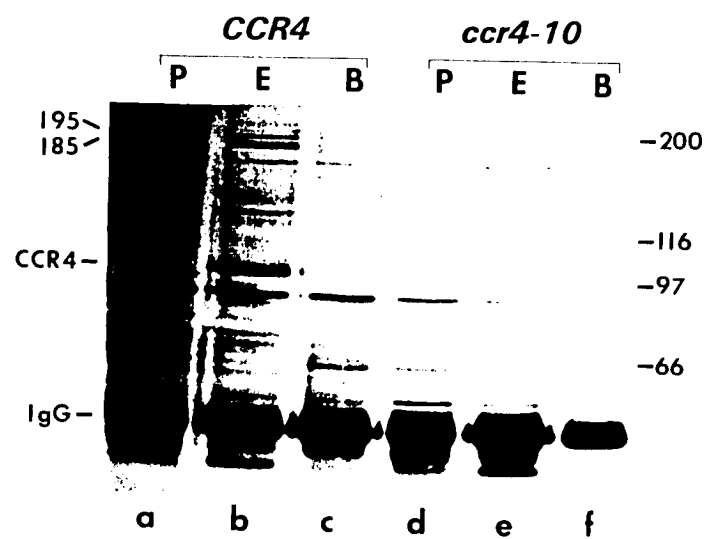


Figure 6B.

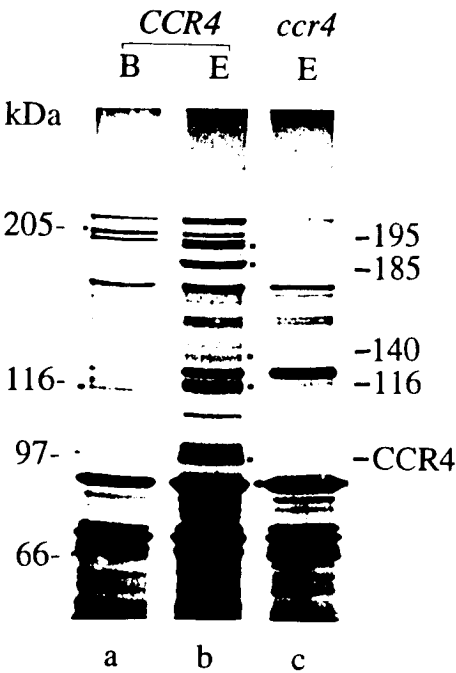


Figure 7.

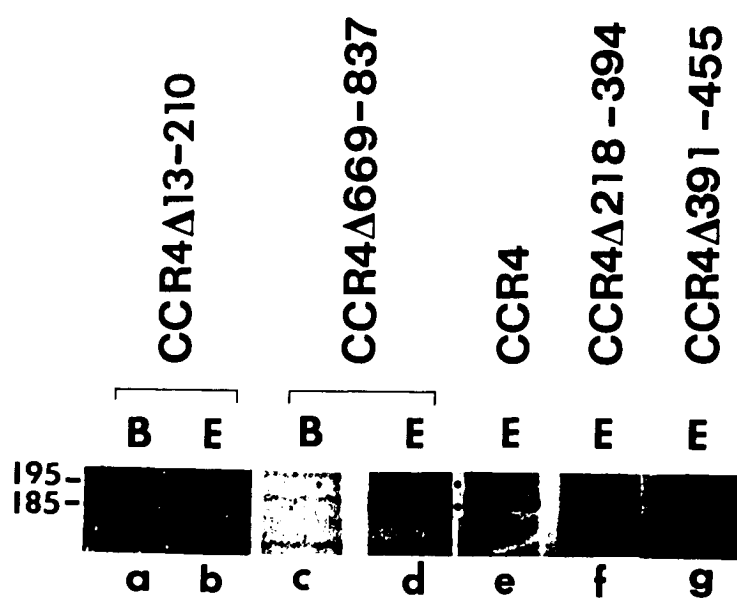


Figure 8.

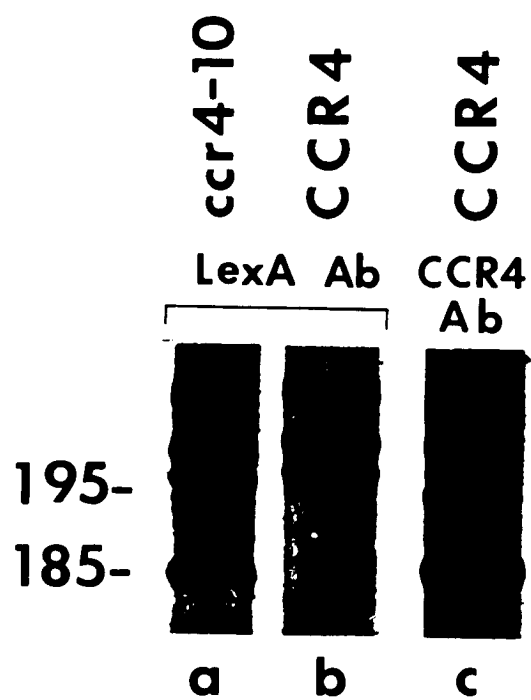


Figure 9.

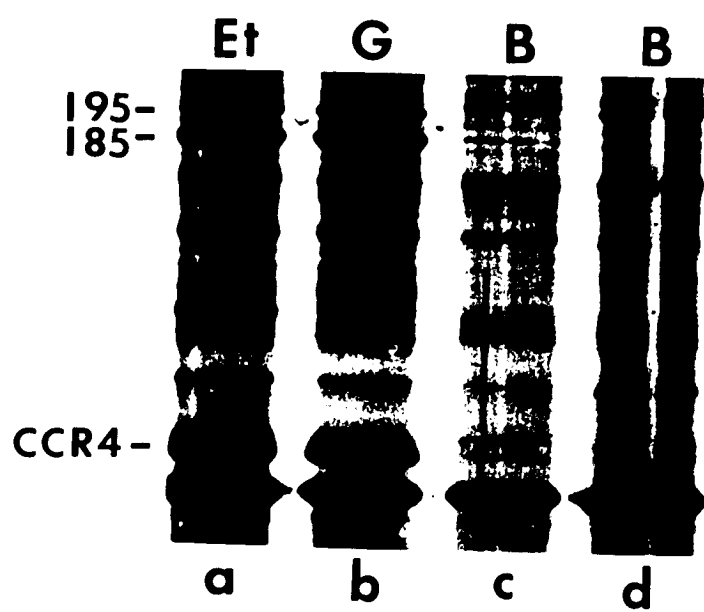


Figure 10.

